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GRANT NUMBER: DAMD17-94-J-4201

TITLE: Effect of Hormone Replacement Therapies and Dietary Phytoestrogens on the Mammary Gland of Macaques

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REPORT DATE: July 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | July 1996 | Annual (1 Jul 95 - 30 Jun 96) |

**4. TITLE AND SUBTITLE**
Effect of Hormone Replacement Therapies and Dietary Phytoestrogens on the Mammary Gland of Macaques

**5. FUNDING NUMBERS**
DAMD17-94-J-4201

**6. AUTHOR(S)**
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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Bowman Gray School of Medicine
Winston-Salem, NC 27157-1040

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200)*

The purpose of this study was to use histomorphometric and immunohistochemical techniques to study the incidence and characteristics of mammary gland hyperplasia, dysplasia and other possible indicators of breast cancer risk, in cynomolgus macaques given long-term hormonal treatments. Treatments evaluated to date include conjugated estrogens (CEE), medroxyprogesterone acetate (MPA), the combination of CEE and MPA, tamoxifen, estradiol (E2), 17α-dihydroequilenin (DHEN), soybean phytoestrogens (SBE), and SBE + E2. Pathologic evaluation, histomorphometric evaluations, and staining for estrogen receptor, progesterone receptor, and the proliferation marker Ki-67 MIB were done. Results are as follows: The addition of MPA to CEE therapy increases, rather than decreases, mammary gland proliferation. Tamoxifen treatment does not induce mammary gland proliferation beyond that seen in controls; this is in contrast to a marked uterotrophic effect. DHEN does not induce mammary gland or endometrial proliferation, relative to controls and in contrast to E2. Soybean estrogens do not induce mammary or endometrial proliferation when given alone, and exert an antagonistic effect on E2-induced proliferation in both sites. New work includes dietary modulation of hormonal effects on mammary gland, identification of p53expression in CEE-treated macaque mammary gland, and development of whole-mount techniques.

**14. SUBJECT TERMS**
Breast Cancer; hormone replacement therapy; mammary gland; proliferation; steroid receptors; conjugated estrogens; medroxyprogesterone acetate; tamoxifen; phytoestrogens

**15. NUMBER OF PAGES**
70

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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J Mark Cline 7/29/96

PI - Signature Date

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## INTRODUCTION

**•Nature of the problem**

Postmenopausal estrogen replacement has been shown to have major beneficial effects in the prevention of coronary heart disease (Avila, 1990; Bush 1987; Henderson 1988; Hunt 1987; Petitti 1986; Stampfer 1985) and osteoporosis (Ettinger 1985; Kiel 1987; Weiss 1980; Ravnikar 1992; Colditz 1990). Unfortunately, the public health impact of these benefits to postmenopausal health has been small because of poor patient compliance. In the United States, 15% of naturally postmenopausal women 45 to 54 years of age use hormone replacement therapy (HRT), while the number decreases to 6% at ages 55 and older. A recent review of this problem indicates that **concern over the risk of breast cancer is the greatest single disincentive for the use of HRT** (Ravnikar 1992). Identification and quantification of this risk is compromised by a lack of basic knowledge of the hormonal regulation of the breast.

Oral contraceptive use is generally considered to have minimal effect on breast cancer risk (WHO, 1992), although there is some evidence for increased risk in long-term current users (La Vecchia, 1995).

Studies of breast regulation, particularly in the postmenopause, are limited. Most studies have involved one of the following:
1. in vitro models which do not adequately mimic the hormonal milieu of the breast
2.in vivo studies in rodent models which are different in many ways from women
3. observational and epidemiologic studies of women which are confounded by lack of experimental rigidity.
4. studies in women using minimally invasive techniques such as fine-needle aspiration, which do not allow study of spatial/paracrine relationships within the breast.

Experimental studies using human tissues are subject to confounding errors: The apparently normal breast tissue in breasts operated for benign or malignant lesions may be subject to paracrine influence from the tumor, and the breasts of reduction mammoplasty operated women contain considerably more adipose tissue than the breasts from normal average women. Fine needle aspiration biopsies from healthy women also have limitations: Different epithelial areas of the mammary gland can not be safely distinguished in such samples. The ideal model would be based on surgical samples from healthy women, which is a practical impossibility.

**Thus the problem lies in the lack of an adequate experimental system in which to test hypotheses relating to breast cancer risk. Our work is designed to use the cynomolgus macaque model to answer questions relating to breast regulation and breast cancer risk.**

**•Background of previous work**

The concerns of women regarding breast cancer risk associated with hormonal therapies have some basis in the results of recent epidemiologic studies (Colditz 1992; Colditz 1993; Coope 1992; Pike 1993). Colditz et al found a significant association between current estrogen replacement therapy and increased breast cancer risk (Colditz 1992, 1993). The mechanistic basis for this increased risk is unknown. The mitogenic effects of estrogens on both breast and endometrial tissue are well recognized, as are the beneficial effects of progestins on endometrial cell proliferation and cancer risk. The action of progestins on breast tissue is more controversial; the literature offers a number of conflicting results both *in vitro* (Mauvais-Jarvis 1986; Haslam 1988; Papa 1990; Moore 1991), and in vivo (Anderson 1989; de Lignieres 1992; Gompel 1993; Bergkvist 1989; Ohi 1992). The assumption that breast and uterus are regulated similarly leads to the conclusion that the combined hormone replacement therapy designed to decrease the risk of endometrial cancer (i.e. estrogen + a

progestin) is also appropriate for breast. However, a recent meta-analysis of studies including women treated with estrogen plus a progestin did not show a protective effect of the use of progestin (Colditz 1993). Recently, data from the Nurse's Health Study has shown a similar result (Colditz 1995).

The effects of various hormone therapies on atherosclerosis and osteoporosis in female monkeys have been studied for some time at our institution (Adams 1991, Clarkson 1989). The work described herein is an attempt to further utilize this model system for study of the breast. Several studies currently underway offer a unique opportunity for evaluation of the effects of estrogens, progestins, and androgens on mammary gland and endometrium *in vivo*, using animals given doses equivalent to those used in women. Therapies used include postmenopausal estrogen (Premarin) with and without a progestin (MPA); postmenopausal treatment with Premarin, MPA, the combination, or tamoxifen; premenopausal administration of Triphasil; premenopausal Triphasil followed by postmenopausal HRT; and postmenopausal nandrolone, an androgenic steroid. Studies are also in progress to assess the effects of dietary (soy) phytoestrogens. This collection of material represents a unique resource for the study of proliferative lesions induced in target tissues by hormonal replacement therapy.

Very few studies have dealt with the responses of macaque mammary glands to exogenous hormones (Speert 1948, Tavassoli 1988) Findings to date are that estrogens, progestins, and growth hormone can induce mammary gland proliferation, and that high doses of estrogens may induce neoplasms (Tavassoli 1988).

**•Purpose of the present work**

The specific aim of this work is to assess the effects of oral contraceptives and hormonal therapies on the incidence and severity of hyperplastic and dysplastic lesions in the mammary gland of macaques. The following hormone therapies are being evaluated.

Treatments given to premenopausal animals
Triphasil
Ethinyl estradiol (the more estrogenic component of Triphasil)
Levonorgestrel (the more progestinic component of Triphasil)
Soybean Phytoestrogens

Treatments given to postmenopausal animals
Premarin (conjugated equine estrogens)
Premarin + medroxyprogesterone acetate
Medroxyprogesterone acetate
Nandrolone
Tamoxifen
17$\alpha$- dihydroequilenin
Soybean Phytoestrogens

**•Methods of approach**

Our basic approach is the use of intermediate markers of breast dysregulation in macaques (hyperplasia, dysplasia, epithelial proliferation measured by Ki-67 expression, and changes in estrogen and progesterone receptor expression) in order to identify which hormonal treatments might induce a greater risk of breast cancer in women. The associations between proliferative breast lesions and breast cancer risk are quite strong in women. Within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation result in increased risk of transformation to the neoplastic phenotype (Cohen 1991, Butterworth 1992). Among women with benign breast lesions, ductal hyperplasia with atypia is a strong risk factor for the development of overt breast carcinoma (London 1992, Page 1988, DuPont 1985). Within populations of cells *in vitro* and *in*

*vivo*, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well (Moolgavkar 1980).

Macaques are similar to women in many aspects of reproductive physiology and anatomy. Macaques have a distinct menarche and menopause, at about 3 and 20 years of age, respectively. They have a 28-day menstrual cycle, with a hormonal profile similar to that of women (Mahoney 1970). Their endometrial responses to endogenous and exogenous hormones parallel those of women (Kaiserman-Abramof 1989). Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar (Schultz 1937, MacPherson 1974, Benirschke 1978). Primate mammary gland has unique cytokeratin phenotype which is identical in macaques and women, but is not shared by more distant species (Tsubura 1991). In this and othe aspects of breast biology relevant to cancer risk (such as proto-oncogene and tumor suppressor gene expression), the mammary gland of macaques is quite similar to that of women (Cline, unpublished data). Mammary neoplasms are uncommon in macaques (Benirschke 1978, Warner 1979). We believe that the female macaque model provides a unique alternative for measurement of responses of the breast to exogenous and endogenous hormones. This model also allows for extensive studies of treatment effects in normal breast which could not be done in women, for example serial biopsy studies or determinations of regional variations in breast regulatory processes.

## BODY

### •Experimental Methods

*Study Design*

Animals with a variety of hormonal manipulations are included in this work, as well as control monkeys from these studies, which allows concurrent study of the normal menstrual cycle. Studies from which tissues are being collected include the following:

**Three studies dealing with postmenopausal hormone replacement therapy:**
88-14 Estrogen replacement/secondary intervention trial
91-20 Estrogen replacement primary intervention trial
93-16 17α-dihydroequilenin study

**One study using premenopausal (contraceptive) steroids and postmenopausal comparison of estrogen replacement versus dietary soy supplementation:**
91-12 Oral contraceptive atherosclerosis primary prevention trial/ Soy as an estrogen alternative trial

**One study of contraceptive steroids alone:**
91-24 Oral contraceptive study (Triphasil components trial)

**One study of androgenic steroids used to prevent osteoporosis:**
92-04 Anabolic steroid study

*The above-listed studies were described in the original grant application. Additional opportunities arising during the past 2 years also include three very exciting studies of potential estrogen antagonists,* namely:

**Two studies of soybean phytoestrogens, given alone and in combination with estradiol:**
93-18 Effects of Soybean Estrogens in peripubertal macaques
94-33 Interactions of Mammalian and Plant Estrogens

**One study of concurrent administration of estradiol and tamoxifen:**
95-13 Interactions of Estrogen and Tamoxifen

A brief description of each study is given below.

**Experiment 88-14**

**Estrogen replacement/secondary intervention trial**

Study design

Progression phase - surgically postmenopausal, adult female monkeys were fed an atherogenic diet, to allow progression of atherosclerosis and osteoporosis for 22 months.

↓

Animals were then randomized into 3 groups:
Ovariectomized control (n = 26)
Premarin (n = 22)
Premarin + Cycrin (n = 21)

↓

Treatment phase (24 months)
Diet changed to low fat and low cholesterol

↓

Euthanasia and necropsy
Termination date: July, 1993

↓

Assessment of:

**Benefits**: Atherosclerosis regression, arrest of osteoporosis progression.

**Risks**: Hyperplastic, dysplastic or neoplastic lesions in breast and endometrium. In particular, the relative effect of Premarin and Premarin + MPA has been assessed.

**Experiment 91-20: Primary intervention trial**

Termination date: April 1995

Study design

Surgically postmenopausal, female cynomolgus monkeys

↓

Randomization to 5 groups:
- Ovariectomized control (n = 15)
- Premarin (n = 15)
- Cycrin (n = 15)
- Premarin + Cycrin (n = 15)
- Tamoxifen (n = 15)

↓

Treatment - fed moderately atherogenic diet, 35 months

↓

Euthanasia and necropsy

↓

Assessment of:

**Benefits**: Cardioprotective effect of Premarin alone and with Cycrin. Direct comparison of cardioprotective effect of Premarin and Tamoxifen.

**Risks**: Hyperplastic, dysplastic, and neoplastic lesions in breast and endometrium. This study is of particular interest because a) it allows comparison of the effects of Premarin and Premarin + MPA with MPA alone, and b) it provides an opportunity to examine the effect of Tamoxifen on normal mammary gland.

**Experiment 93-16: 17α-dihydroequilenin study**
Termination date: December 1993

Study design

Young, female rhesus monkeys

↓

Randomization to 3 groups:

Cycling control (n = 16)
Ovariectomized control (n = 17)
17α-dihydroequilenin (n = 17)

↓

Treatments were given for 21 weeks. Animals received a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Assessment of:

**Benefits**: Cardioprotective effect of 17α-dihydroequilenin.

**Risks:** Mammary hyperplasia, dysplasia, and neoplasia relative to either control group.

**Experiment 91-12**
**Atherosclerosis/osteoporosis primary prevention trial**
Termination date October 1993 (interim) and December 1996

Study design (phase I)
Premenopausal, female cynomolgus monkeys

↓

Randomization to 2 groups:
Control (n = 100)
Triphasil (n = 100)

↓

Treatment phase - Animals are fed a moderately atherogenic diet and treated for 24 months.

↓

Interim arterial biopsy

↓

Assessment of:
Benefits - cardioprotective effect of premenopausal estrogen use, particularly for stressed females.
Increase in peak bone mass from premenopausal estrogen use.

↓

Study design (phase II)

Surgically postmenopausal monkeys

↓

Randomization of the two groups from part I (estrogen use or not) into three groups:
1) Control (n = 63)
2) Premarin (n = 63)
3) Soy phytoestrogen (n = 63)

for a treatment period of 36 months.

↓

Final necropsy

↓

Assessment of:

**Benefits**: Does premenopausal estrogen use add to the postmenopausal hormone replacement therapy effects on atherosclerosis and osteoporosis?

**Risks**: Hyperplasia and dysplasia of target tissues.
Possible protective effect of premenopausal contraceptive use on endometrium, and possible increased mammary tumor risk
What are the uterotrophic and mammotrophic effects of phytoestrogens?

**Experiment 91-24**
**Atherosclerosis/contraceptive steroids primary prevention trial**
Termination dates: June 1993 (interim sacrifice) and fall 1995

Study design

Premenopausal, female cynomolgus monkeys

↓

Randomization to 4 groups:

Control (intact, normally cycling)
Triphasil (n = 24)
Cyclic ethinyl estradiol (n = 24)
Cyclic levonorgestrel (n = 24)

↓

Treatment was given for 35 months. Animals received a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Assessment of:

**Benefits**: Cardioprotective effect of premenopausal estrogen use.

**Risks**: Mammary hyperplasia, dysplasia and neoplasia, and whether such effects relates to the ethinyl estradiol or levonorgestrel component of Triphasil.

**Experiment no. 92-04**
**Osteoporosis primary prevention trial**
Termination date: June 1994

Study design

Pre- and postmenopausal female cynomolgus monkeys

Randomization:

Cycling control (n = 15)
Ovariectomized control (n = 15)
Nandrolone in year 1 after ovariectomy (n = 15)
Nandrolone in year 2 after ovariectomy (n = 15)

Treatment was given for 24 months. Animals were fed a moderately atherogenic diet.

Euthanasia and necropsy

**Benefits**: Prevention/treatment of osteoporosis

**Risks**: Coronary artery atherosclerosis exacerbation. Effect of androgenic/anabolic steroids on mammary gland and endometrium.

**Experiment No. 93-18**
**Effects of Soybean Estrogens**
Termination date: January 1995

Study design

Peripubertal female cynomolgus monkeys

↓

Randomization:
Untreated controls (n = 13)
Soybean estrogens (n = 14)

↓

Treatment was given for 12 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

**Benefits**: Prevention/treatment of atherosclerosis.

**Risks**: Potential adverse estrogenic effects of soybean estrogens, such as induction of mammary or endometrial proliferation.

**Experiment No. 94-33**
**Interactions of Mammalian and Plant Estrogens**

Termination date: October 1995

Study design

Postmenopausal female cynomolgus monkeys

↓

Randomization:
- Ovariectomized control (n = 15)
- Estradiol (n = 15)
- Soybean estrogens (n = 15)
- Estradiol + Soybean estrogens (n = 15)

↓

Treatment was given for 6 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

**Benefits**: Prevention/treatment of atherosclerosis; potential additive effect of soy and estradiol in the prevention of arterial and bone disease. Potential antagonistic effect of soy and estradiol, which might prevent breast and endometrial proliferation caused by estradiol.

**Risks**: Potential antagonistic effect of soy and estradiol, which might reduce the effectiveness of coronary artery protection. Potential additive effect of soy and estradiol on mammary gland and endometrium, resulting in increased proliferation and cancer risk.

**Study No. 95-13**
**Interactions of Estrogen and Tamoxifen**

Termination date: June 1996

Study design

Postmenopausal female cynomolgus monkeys

Randomization:
Ovariectomized control (n = 6 )
Estradiol (n = 6 )
Estradiol + tamoxifen (n = 6 )

Treatment was given for 2 months. Animals were fed a moderately atherogenic diet.

Euthanasia and necropsy

**Benefits**: Protective effect of tamoxifen on breast, which will presumably be reflected in lower proliferation in breast .

**Risks**: Endometrial proliferation induced by tamoxifen and estradiol, leading to hyperplasia and increased risk of neoplasia.

*Diets/Drug Dosing*
The hormones were administered twice daily in the diet, with the exception of nandrolone. Most animals consume a moderately atherogenic diet (40% of calories from fat, 0.2 mg of cholesterol per Calorie). Monkeys are fed approximately 120 Calories per kg of body weight per day. Doses were as follows:

| Drug | Abbreviation | Dose equivalent per woman per day |
|---|---|---|
| Conjugated equine estrogens | CEE | 0.625 mg |
| Medroxyprogesterone acetate | MPA | 2.5 mg |
| 17β-Estradiol | E2 | 2 mg |
| Tamoxifen | TAM | 20 mg |
| Ethinyl estradiol | EE | Days 1-6: 0.03 mg<br>Days 7-11: 0.04 mg<br>Days 12-21: 0.03 mg<br>Days 22-28 : no drug |
| Levonorgestrel | LN | Days 1-6: 0.05 mg<br>Days 7-11: 0.075 mg<br>Days 12-21: 0.125 mg<br>Days 22-28: no drug |
| 17α-Dihydroequilenin | DHEN | 0.312 mg/kg |
| Soybean estrogens | SBE | 99.7 mg |
| Nandrolone | - | No daily equivalent; animals were given injections of 25 mg nandrolone decanoate by intramuscular injection every 3 weeks. |

Drug doses were computed as:
human dose divided by 1800 Calories/woman/day = dose per Calorie of diet
*Doses arrived at by this means were therefore consistently scaled, and adjusted for metabolic rate. They are similar to the dose calculated by scaling on the basis of body surface area (Mordenti 1986).*

*Tissue collection*
Mammary glands were collected at the end of each study, when all monkeys are euthanized and necropsied. Tissues were fixed in 4% buffered paraformaldehyde at 4°C. The tissue was removed from paraformaldehyde after 24 hours, stored in 70% ethanol at 4°C, and later trimmed to 3 mm in thickness, embedded in paraffin using standard histologic procedures, and sectioned at 5 μm for immunostaining.

*Histopathology*
Mammary gland slides were subjectively classified as atrophic, hyperplastic, or neither. The treatment group of each animal was obscured during the procedure to prevent observer bias. Hyperplasia, atypia, cystic lesions, and secretory activity were noted. Lesions were independently graded as none, mild, moderate or severe. The criteria of the World Health Organization were used for tumor classification (WHO, 1982)

*Morphometry*
Mammary gland thickness, mammary lobular size, and area fraction of the mammary tissue occupied by glands are measured from histologic sections using video microscopy and a MacIntosh computer-based, public-domain image analysis program (NIH Image).

*Stereology*
In early studies prior to acquisition of the image analysis system, estimates of the relative proportions of tissue components in the mammary gland were made by point counting, after the method of Chalkley (Chalkely, 1945). These included percentage of gland occupied by epithelium, connective tissue and fat. Numbers of points intercepting each lobule were also recorded, as a relative indicator of lobular size.

*Sex steroid receptors and proliferation marker staining methods.*
Staining procedures were done on fixed, paraffin-embedded tissues. The basic staining procedure uses an avidin-biotin-peroxidase method (Wordinger 1987) modified for antigen retrieval from paraffin-embedded tissue. The estrogen receptor and progesterone receptor analyses were performed with reagents supplied by Dako laboratories (Dako Corporation, Carpinteria, CA, USA), and Immunotech laboratories (Immunotech, Marseille, France), respectively.

*Assessment of proliferation (Ki67-MIB)*
We use the KI-67 MIB-1 (MIB) monoclonal antibody (Immunotech, Marseille, France) that gives an immunostaining identical to Ki-67 antibody and which can be used on paraffin embedded tissue sections (Cattoretti 1992). As for the receptor analysis, the MIB basic staining procedure is done by an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin embedded tissue. The murine monoclonal antibody Ki-67 reacts with a human DNA-binding protein that is present in proliferating cells but absent in quiescent cells. A detailed cell cycle analysis showed that the Ki-67 antigen is expressed in G1, S, G2 and mitosis (with maximum levels during G2 and M phases) but not in G0 and using this antibody an exact determination of the growth fraction of a given human cell population, regardless of whether it is normal or malignant, has been possible (Gerdes 1991).

*Quantification of immunohistochemical staining*
Immunostained cells were quantified by cell counting in sections, by an observer blinded to treatments. Epithelial cells lining the alveoli, the terminal and major ducts were considered separately in order to assess regional differences. Labeled cell nuclei were identified as unlabeled (0), weakly (+), moderately (++), or intensely (+++) labeled. At least 100 cells per slide were counted at 3 different sites for each combination of animal, tissue site and stain type. Major ducts and alveoli were easily identifiable, but clearly defined terminal ducts could not be identified in some cases.

*Development of mammary whole-mount methods*
Mammary gland whole-mounts were prepared by dissecting the mammary fat pad and gland free of the overlying skin, defatting in acetone, clearing in methyl salicylate, hydrating the tissue through graded alcohols, staining with toluidine blue, destaining in methanol, then dehydrating the tissue through graded alcohols and mounting on glass slides.

*Statistical methods*
Statistical analysis is performed using the Mann-Whitney U-test with Bonferroni corrections for multiple comparisons, Kruskal Wallis test, Chi-square test, and Spearman's rank correlation test.

*Ancillary Projects*
Several ancillary projects have been carried out which enhance the understanding of mammary data collected. These include:
1) development of vaginal cytology methods for prospective screening of live macaques for estrogenic effects on the reproductive tract;
2) development of a method to retrospectively approximate the reproductive history of monkeys; and
3) development of a morphometric protocol to assess treatment-related changes in ovaries of oral contraceptive-treated macaques, in order to document treatment effects.

**•Results obtained**

A brief outline of accomplishments to date is followed by a presentation of specific experimental findings. Progress to date includes:

1. Collection of over 500 paired, frozen and fixed breast and endometrial samples, from macaques treated with conjugated estrogens with and without medroxyprogesterone acetate, tamoxifen, triphasic oral contraceptives, nandrolone, estradiol, dietary phytoestrogens, and controls.

2. Development of morphometric and cell counting methods for evaluation of breast and endometrium, including acquisition of a computerized video microscopy/image analysis system and development of standard measurement procedures.

3. Refinement and application of immunohistochemical methods for detection of the proliferation marker Ki-67, estrogen receptors and progesterone receptor; tissues from approximately 300 animals have been stained and evaluated to date.

4. Publication of a manuscript to the American Journal of Obstetrics and Gynecology detailing morphologic and immunohistochemical changes in the breast of surgically postmenopausal macaques given conjugated estrogens with or without the addition of MPA (from study 88-14). The manuscript is included in Appendix A, page 34

5. Results in mammary gland have been compared to endometrial morphology and Ki-67, ER, and PR staining in endometrium, in the same animals used for the above manuscript (via a separate grant, received from the Office of Research on Women's Health). This parallel study was the basis of a Young Investigator Award to Dr. Cline for presentation of the findings at the North American Menopause Society meeting. (Appendix B, page 67)

6. Study of the regional variation in breast regulation, by quadrant and distance from the nipple. Identification of regional variations provides us with an assessment of the degree of random or predictable intrinsic variation in within breast tissue, a parameter of considerable interest should we plan biopsy-based studies in the future. Results of this study have been submitted to the journal Breast; (see Appendix A, page 42).

7. Development of histopathologic criteria for retrospectively distinguishing the uteri of parous and nulliparous macaques. Since some animals in our studies were acquired as adults with an unknown reproductive history, and parity affects long-term breast regulation, this is an important source of variation in the breast which we needed to identify. This work was presented at the 1995 meeting of the American College of Veterinary Pathologists (Appendix B, page 68), and a manuscript is in preparation for submission to the journal Veterinary Pathology.

8. Vaginal cytologic studies demonstrated classical estrogenic effects of estradiol and conjugated estrogens in macaques, a weak estrogenic effect of tamoxifen on the vagina, and no estrogenicity of soybean estrogens in the vagina. These results were published in the journal Fertility and Sterility (Appendix A, page 61).

9. Ovarian histomorphometric studies identified distinct atresia-inducing effects of oral contraceptives, including progestin-only contraceptives, in follicles of treated animals.

10. Whole-mount methods for assessing mammary gland development have been developed in our laboratory; based on preliminary examination of a few samples, we believe that this method will provide an important adjunct to our existing procedures for evaluating mammary gland proliferation. In particular, the degree of differentiation of the gland is best assessed by this

method.

11. Our demonstrated interest in dietary chemoprevention of breast cancer has led to the generation of an invited review of the potential chemopreventive properties of phytochemicals (Cline 96).

The following are the specific technical objectives proposed for 1994/1995 and 1995/1996, accompanied by a report of what has been accomplished.

| **Technical Objective** | **Work Accomplished** |
|---|---|
| *Year 1 (1994/1995)* | |
| Processing, staining, and measurement from tissues collected in 1993; studies 88-14 (final sacrifice), 91-24 (interim sacrifice), 91-12 (interim sacrifice). | Completed. |
| Collection of tissues from studies 92-04 and 93-16. | Completed. |
| *Year 2 (1995/1996)* | |
| Collection of tissues from studies 91-20, 91-24, and 91-12 (final sacrifices). | Collections have been made from 91-20 and 91-24. Collections from 91-12 have been delayed by a change in the design of the parent study. |
| Processing, staining, and measurement of tissues collected. | This work is in progress as planned. |
| Presentation and publication of interim results from studies 91-24 and 91-12, final results from study 88-14. | Final results from studies 88-14 (CEE+/-MPA) and 93-16 have been published. Preliminary data from experiments 94-33, 91-20, 93-18, and 91-24 have been presented. Interim endpoints from experiment 91-12 have been published (vaginal cytology), but mammary gland evaluation awaits tissue collection. |

*Specific Results Listed By Study*

Substantial results are available from three studies, and are described below. Ancillary projects relevant to this work are described at the end of the results section.

**I. Conjugated Estrogens with or without MPA (Study 88-14)**
This is a long-term comparison of the effects of CEE and CEE+MPA in surgically postmenopausal macaques (the study design is shown on page 9). Results were described extensively in last year's progress report, and will not be repeated here as they are included in Appendix A (page 34).

The most relevant finding in this study was that the addition of the progestin MPA to estrogen (CEE) treatment had differing effects on the mammary gland and endometrium of macaques: That is, MPA antagonized the proliferative effect of CEE in the endometrium, but not the mammary gland. In fact, the addition of MPA to CEE treatment *increased* prolifertion in the mammary gland.

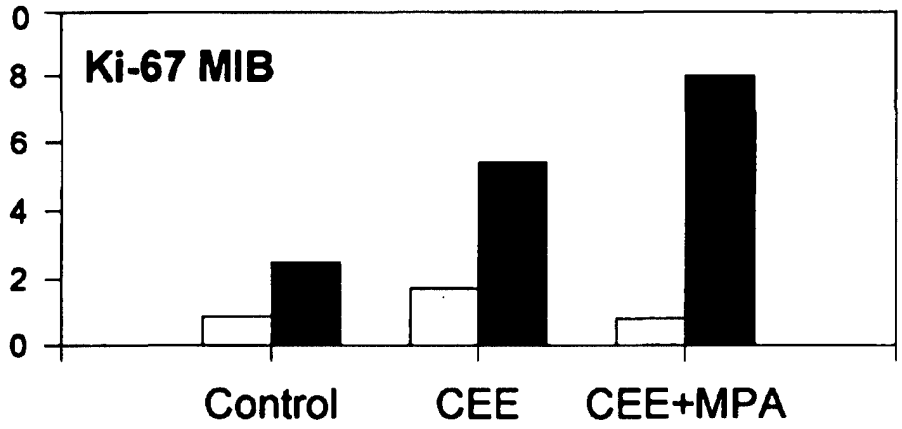


Figure 1. Divergent effects of combined estrogen/progestin treatment on the endometrium (white bars) and mammary gland (black bars) of macaques. Bars indicate the percentages of cells labeled with the proliferation marker Ki-67.

**II. Conjugated estrogens, MPA and Tamoxifen (Study 91-20)**
This is a recently terminated study of the comparative effects of CEE, MPA, CEE+MPA, and tamoxifen in surgically postmenopausal macaques (the study design is shown on page10).

In this study, results in the groups given CEE, and CEE+MPA, are similar to those in the preceding experiment. Proliferation data on these groups, and the additional groups given MPA alone and tamoxifen, are shown in Figure 2. Again, it is apparent that CEE+MPA exerts a greater mammotrophic effect than CEE alone, in contrast to the findings in the uterus. As might be expected, tamoxifen does not cause an increase in mammary glandproliferation.

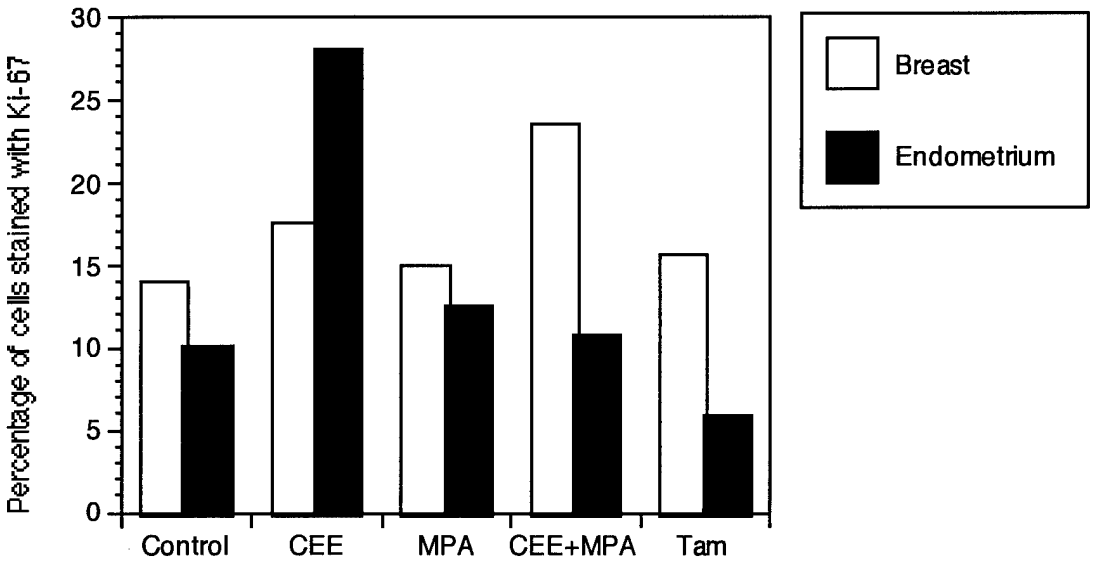


Figure 2. Comparison of mammary gland and endometrial thickness in animals given CEE, MPA, or Tamoxifen. White bars = percentage of cells stained postively with the proliferation marker Ki-67 in mammary glands. Black bars = Percentage of cellstained positively with the proliferation marker Ki-67 in epithelial cells of the uterine zona functionalis.

Further details of this study are given in the abstract presented to the American Association for Cancer Research (Cline 1996), in Appendix B (page 69). An assessment of combined tamoxifen and estradiol is in progress (Study 95-13, page 17)

**III. DHEN versus Conjugated Estrogens (Study 93-16)**

This is a study of the relative effects of 17α-dihydroequilenin (DHEN) in pre- and post-menopausal macaques (the study design is shown on page 11). DHEN has considerable potential for use as a "selective" estrogen, exerting beneficial effects on the cardiovascular system and bone without producing increased breast and endometrial proliferation. In this study, DHEN did not exert mammotrophic or uterotrophic effects. This data was given in last year's report, and are summarized in Figure 3.. Endometrial data from this study have been published (Washburn 1996).

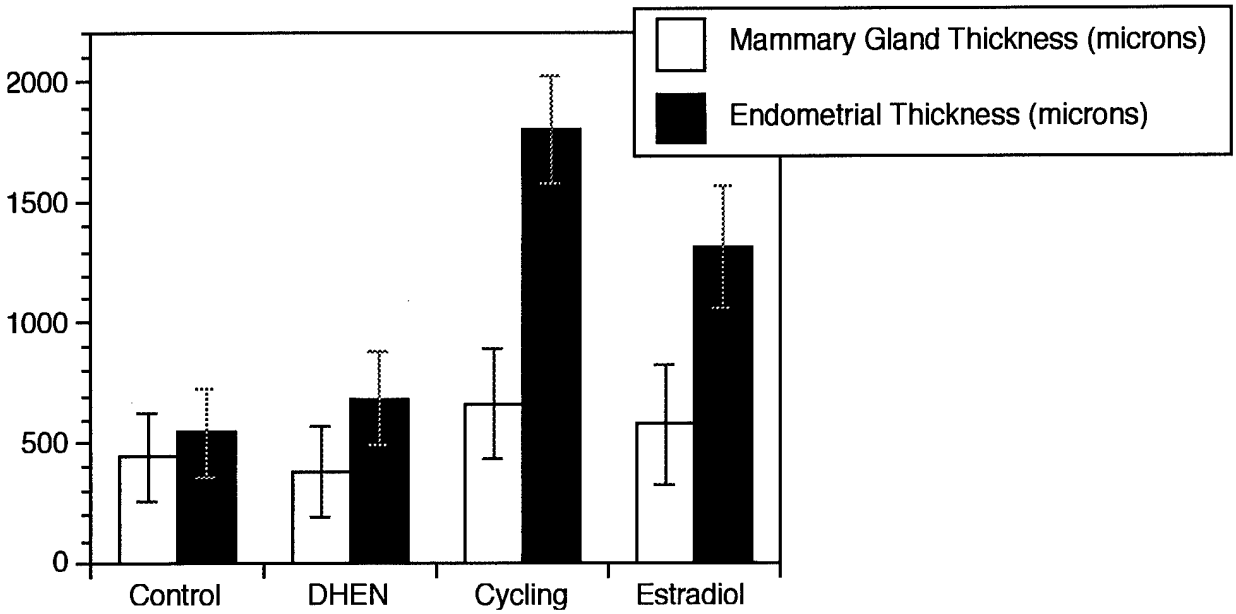


Figure 3. Dihydroequilenin does not induce proliferation in mammary gland or endometrium of macaques (not statistically different from controls) .

**IV. Triphasic Oral Contraceptives (Experiment 91-24)**

This study seeks to explore the possible role of triphasic oral contraceptive use on chronic disease risk in the monkey model (see experimental design, page13). A particularly interesting aspect of this study is the concurrent evaluation of typical triphasic oral contraceptives (modeled after the widely used Triphasil ™), and the individual components of the contraceptive, namely ethinyl estradiol and levonorgestrel. To date, no statistically significant treatment-related differences have been identified in mammary glands of animals treated with the whole preparation or its components.

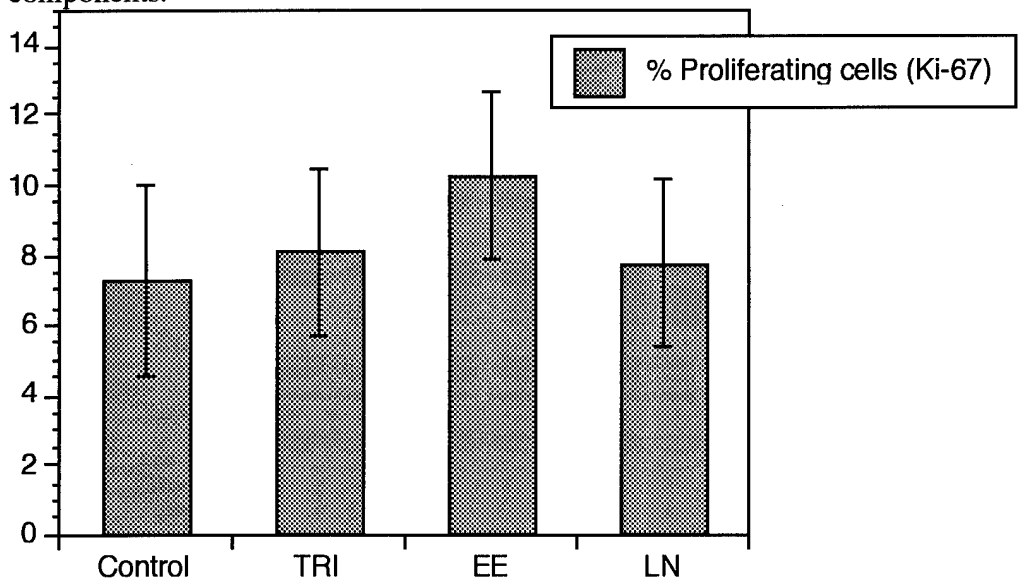


Figure 4. Effects of Triphasil (TRI), ethinyl estradiol (EE) and levonorgestrel (LN) on proliferation in lobuloalveolar tissue of the macaque mammary gland. There are no statistically significant differences.

It is of interest to contrast this study with experiment 88-14, and in particular to note that the

combination of an estrogen and a progestin does not increase epithelial proliferation in these premenopausal animals. This reflects the biology of breast cancer risk in women, in which oral contraceptive use does not produce profound changes in breast cancer risk. Possible sources of this difference include 1) differences between breast regulation in pre- and post-menopausal animals; 2) differences due to the specific estrogen and progestins used; or 3) differences resulting from cyclic, as opposed to continuous, administration of hormones.

**V. Effects of Soy Phytoestrogens on Peripubertal Macaques (Experiment 93-18)**
Our recent work with dietary modulation of intermediate markers of cancer risk has produced some intriguing results. This first of our soy studies was done as a pilot project in a small cohort of female monkeys fed soy phytoestrogens, and demonstrates (as do the vaginal cytology data) that soy phytoestrogens do not induce increases in mammary or endometrial proliferation, as estimated by morphometric measurement of the percentage of each tissue made up of epithelial cells.

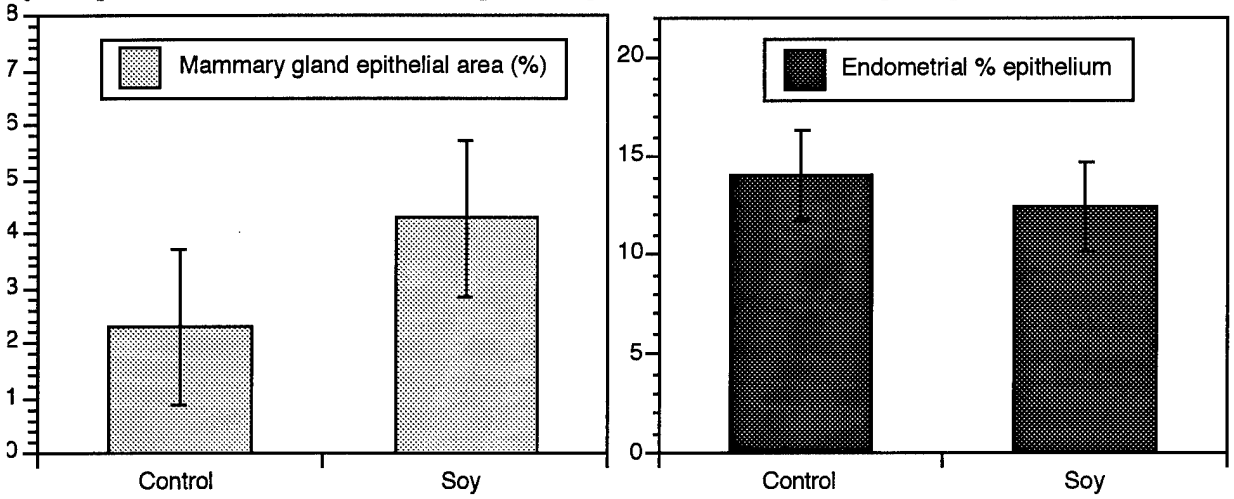


Figure 5. Soy phytoestrogens do not induce mammary gland or endometrial hyperplasia.

**VI. Interactions of Mammalian and Plant Estrogens (Experiment 94-33)**
Further explorations of soy effects on mammary gland led us to examine the effects of estradiol in concert with dietary soy supplementation. The study design is outlined on page 16 Findings to date indicate that soy phytoestrogens are not themselves classically "estrogenic" (i.e. proliferation - inducing) in mammary gland or endometrium.

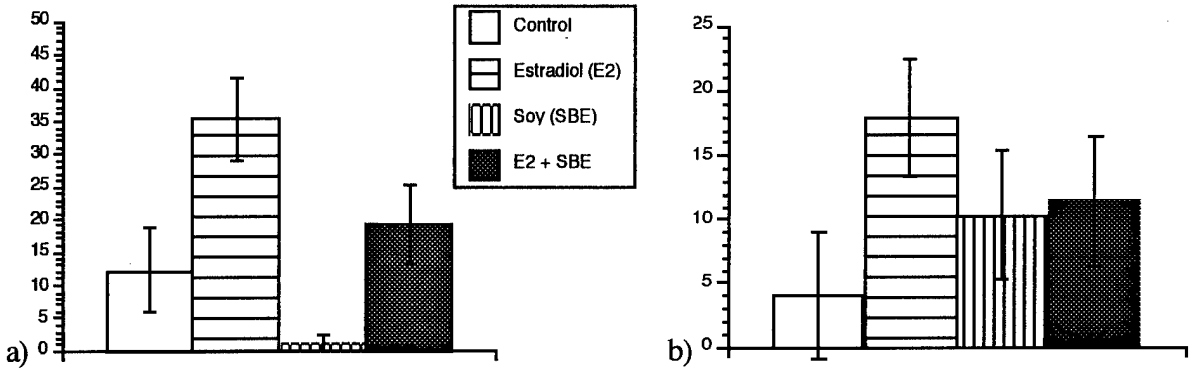


Figure 6. - Immunohistochemical staining for the proliferation marker Ki-67 in a) endometrium and b) mammary gland of cynomolgus macaques. Estrogen-induced proliferation is antagonized by the addition of soybean estrogens (SBE). Soy phytoestrogens do not induce mammary gland

proliferation alone, and exert an antagonistic effect on estrogen-induced proliferation. Only the estrogen-treated group differs from controls at $p < 0.05$.

Figure 7.

Further details of this study are given in the 1996 AACR abstract included in Appendix B, page 69.

**VII. Application of Novel Markers to the Monkey Model**

During the course of this grant, we have explored a number of potential intermediate markers of breast cancer risk, including epidermal growth factor receptor, c-erbB-2, p53, and IGF-2. Of these, p53 expression in estrogen-treated animals is the most clearly elevated. Work done in collaboration with our colleagues at the Karolinska Institute in Stockholm has shown that wild-type p53 is up-regulated in macaques and can be detected using commercially available antibodies. We have done some preliminary staining of macaque mammary gland tissue for c-erbB-2 and p53. In the case of c-erbB-2, some cell membrane reactivity has been found among estrogen-treated animals in mammary gland epithelium (data not shown). Among the few test cases, those treated with estrogens have a greater proportion of positively stained cellsfor wild-type p53.

Table 1. p53 reactivity in macaque mammary gland.

| Group | Number analyzed | Number of animals with staining | | |
|---|---|---|---|---|
| | | Mild staining | Moderate staining | Marked staining |
| Control | 10 | 0 | 0 | 1 |
| Conjugated estrogens 0.625 mg/day equivalent | 18 | 2 | 6 | 5 |

**VIII. Development of methods for assessment of mammary gland whole mounts.**

Many investigators in the field of mammary gland biology and breast cancer research use the technique of assessing mammary gland whole mounts to assess glandular differentiation (Speert 1948, Squartini 1986, Russo 1990). We have recently adapted methods described for use in humans and rodents to use with our tissues, and intend to proceed with further characterizing the macaque mammary gland during differentiation, hyperplasia, and the development of preneoplastic lesions.

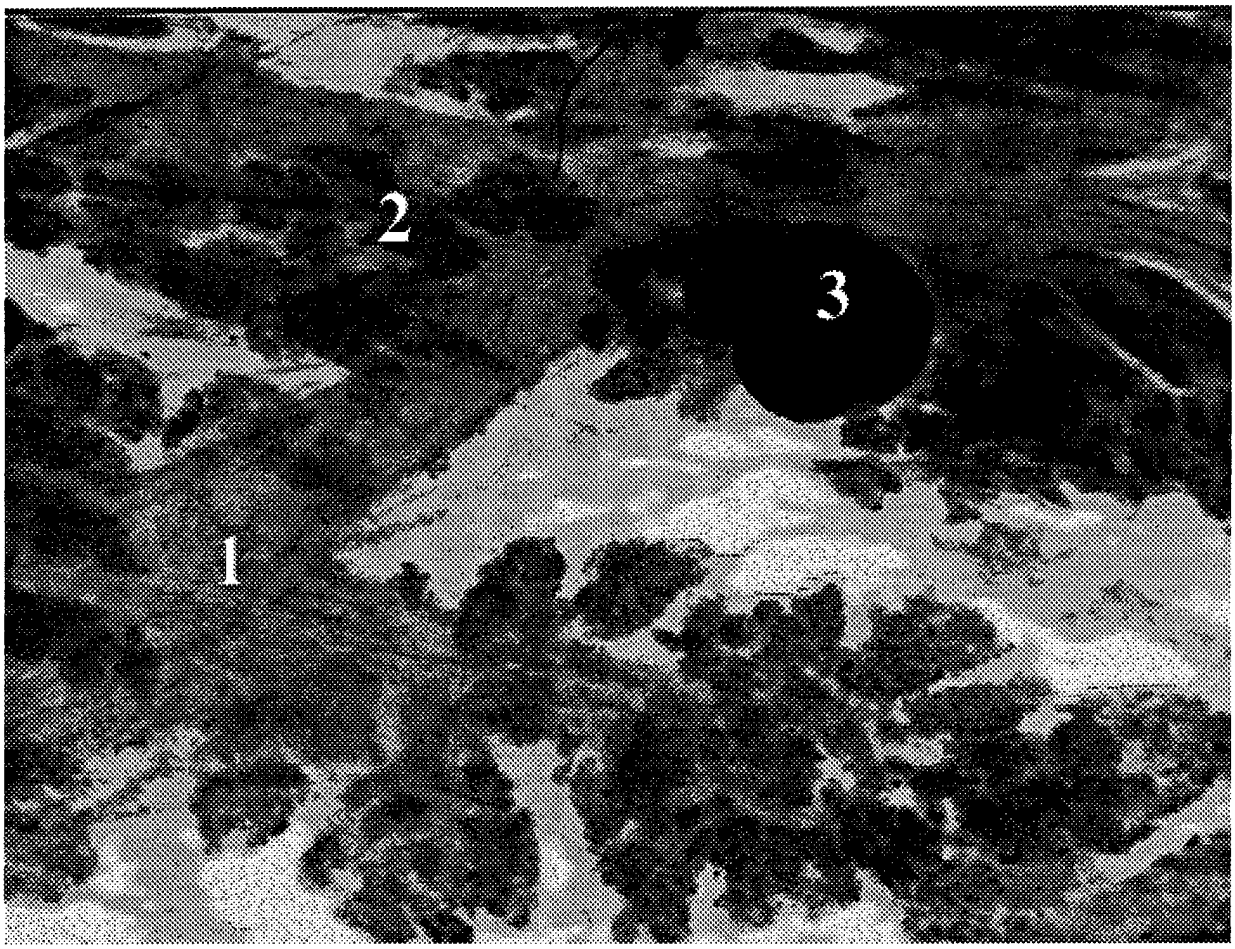


Figure 8. Mammary gland whole mount from an estrogen-treated cynomolgus macaque, demonstrating a large duct (1), extensive lobulolaveolar development (2), and a focal hyperplastic nodule (3).

## Ancillary Projects

*Vaginal cytologic evaluation of hormone-treated animals*
Vaginal cytologic examinations have been made on animals in studies 91-12 during the latter half of the experiment (see page 12 for the study design), and in study 91-20 (the study design is on page10). Results presented in last year's report indicate that CEE and tamoxifen exert an estrogenic effect on the vaginal epithelium, and that SBE does not. These results are described in detail in the paper published this year in Fertility and Sterility (Appendix A, page 61)

*Identification of Previously Pregnant animals*
The presence of perivascular extracellular mucinous matrix deposits was identified as an indicator of prior pregnancy. This study was briefly described in last year's report, but has since been finalized and presented. Details of the study are given in the abstract presented to the American College of Veterinary Pathologists, in Appendix B (page 68)

*Assessment of oral-contraceptive-related changes in monkey ovaries*
This project was undertaken by a summer student in my laboratory, and was not funded by this grant; nonetheless, it has produced interesting data indicating that oral contraceptives do in fact alter the ovarian function of macaques at the doses given. This work has resulted in beneficial collegial

interactions between investigators at Bowman Gray, Duke University, and the National Institute of Environmental Health Sciences.

**•Discussion of results relative to goals of the research**

The data presented herein clearly demonstrate that the macaque mammary gland can be used to provide a model of breast regulation in the post-menopausal period. This model is unique in that it provides an in vivo assessment of hormone effects on the primate breast, and can also be used to make comparisons of breast and endometrial effects in the same subject.

Several significant findings have been made to date. In the first year of this grant, we observed that the addition of MPA to conjugated estrogens did not result in suppression of the mammotrophic effects of CEE, but instead in a greater proliferative response than is seen with CEE alone. This finding addresses precisely the type of question the project is designed to target, providing a result which is of great relevance to public health, but which can only be explored with great difficulty in human subjects. This is in agreement with recent reports such as that of the Nurses' Healt Study (Colditz 1995).

During the second year, we made the observation that dietary soy supplementation has the potential to protect the mammary gland from the tumor-promoting proliferative effects of estrogens. This finding has broad implications for the use of dietary modulation of breast cancer risk.

## CONCLUSIONS

### General Summary:

Technical objectives outlined in the initial application have been met on schedule, with only minor changes.

The morphometric and immunohistologic methods proposed in the initial application have been applied successfully to a number of mammary gland samples from macaques.

Initial results have been accepted for publication in a peer-reviewed journal, and have resulted in an award from the North American Menopause Society to the principal investigator.

### Specific Conclusions:

In surgically post-menopausal cynomolgus macaques,

The addition of MPA to CEE therapy increases, rather than decreases, mammary gland proliferation. This finding is in contrast to the uterus, where MPA antagonizes the proliferation induced by CEE.
Tamoxifen treatment does not induce mammary gland proliferation beyond that seen in controls; this is in contrast to a marked uterotrophic effect.

DHEN does not induce mammary gland or endometrial proliferation, relative to controls and in contrast to CEE.

Soybean estrogens may be selectively-active compounds which can inhibit the proliferation-inducing effects of estrogens when given in combination with them.

### Recommendations:

A great deal of important information can be gained within the scope of this project as initially written. However, after making the initial observations of hormone effects of these intermediate markers of cancer risk in breast, it will be vital to proceed on to more mechanistic studies of the role of growth factors and growth factor receptor expression in the proliferative response. The continuing controversy over breast cancer risk associated with hormonal therapies, particularly with regard to the role of progestins, indicates a lack of understanding of basic regulatory processes in the breast. The number of mediators potentially involved in breast regulation is large, including epidermal growth factor, insulin-like growth factor, relaxin, prolactin, tumor necrosis factor alpha, and others. The macaque model is ideally suited to the exploration of stromal-epithelial interactions in breast regulation, and this is and area we would like to pursue in future work. We are also excited by the potential for dietary chemoprevention of cancer, and we believe that dietary soy supplementation may be an avenue worthy of further exploration. The anticarcinogenic effects of soy isolates are well-documented (Barnes 95), and we hope to pursue this aspect of the work further.

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**Personnel Receiving Pay:**

| Name | Social Security No. | Role | Percentage of Salary |
|---|---|---|---|
| J. Mark Cline | | Principal Investigator | 25% |
| Brian A. McCollough | | Laboratory Technician II | 50% |
| Shannon Schmotzer | | Laboratory Technician II | 50% |

**Graduate Degrees Received:**
No graduate work was supported by this grant.
Brian McCollough is currently pursuing a Master's Degree in Biology from North Carolina Agricultural and Technical University, Greensboro, NC. He is not receiving stipend support fromthis grant, but the development of immunohistochemical methods of measuring cell proliferation in this grant has formed the basis of his thesis work, entitled. "Immunohistochemical Markers of Cell Proliferation in Macaques" We anticipate awarding of his degree in late 1996.

## APPENDICES

### Appendix A: Publications

(copies of items in **bold** are attached)

Cline JM, Soderqvist G, Skoog L, von Schoultz B. Divergent effects of hormone replacement on mammary and endometrial tissues of macaques. Menopause. 1995;2:278

**Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques. American Journal of Obstetrics & Gynecology. 1996;174:93-100**

**Cline JM, Paschold JC, Anthony MS, Obasanjo IO, Adams MR. Effects of hormonal therapies and dietary soy phytoestrogens on vaginal cytology in surgically postmenopausal macaques. Fertil Steril. 1996;65:1031-1035**

**Cline JM, Soderqvist G, von Schoultz E, von Schoultz B, Skoog L. Regional distribution of proliferating cells and hormone receptors in the mammary gland of surgically postmenopausal macaques. Submitted to Breast.**

Cline JM, Hughes CL. Phytochemicals for the prevention of breast and endometrial cancer. In Biological and Hormonal Therapies of Cancer, eds. H Muss and K Foon. Kluwer Academic Publishers. In press.

Washburn SA, Honoré EK, Cline JM , Helman M, Wagner JD, Adelman SJ, Clarkson TB. Effects of 17$\alpha$-dihydroequilenin sulfate on atherosclerotic male and female rhesus monkeys. Am J Obstet Gynecol, in press.

# Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques

**J. Mark Cline, DVM, PhD,[a] Gunnar Soderqvist, MD,[b] Eva von Schoultz, MD, PhD,[c] Lambert Skoog, MD, PhD,[d] and Bo von Schoultz, MD, PhD[b]**

*Winston-Salem, North Carolina, and Stockholm, Sweden*

**OBJECTIVE:** Our purpose was to define the proliferative response and receptor status in the mammary glands of surgically postmenopausal macaques given hormone replacement therapy, equivalent for monkeys to that given women.

**STUDY DESIGN:** Surgically postmenopausal adult female cynomolgus macaques *(Macaca fascicularis)* were given either no treatment ($n = 26$), conjugated equine estrogens ($n = 22$), or combined therapy with conjugated equine estrogens and medroxyprogesterone acetate ($n = 21$). Drugs were administered in the diet, at doses equivalent on a caloric basis to 0.625 mg per woman per day for conjugated equine estrogens and 2.5 mg per woman per day for medroxyprogesterone acetate, for 30 months. Mammary gland proliferation was assessed subjectively and by morphometric and stereologic means. Estrogen receptor and progesterone receptor content and proliferation were studied by immunohistochemistry.

**RESULTS:** In this model combined therapy with conjugated equine estrogens and medroxyprogesterone acetate induced greater proliferation than did conjugated equine estrogens alone. The percentage of estrogen receptor–positive cells was decreased in the conjugated equine estrogens plus medroxyprogesterone acetate group. The percentage of progesterone receptor–positive cells was increased by treatment with conjugated equine estrogens alone.

**CONCLUSION:** These results indicate a proliferative response of mammary gland epithelium to therapy with conjugated equine estrogens plus medroxyprogesterone acetate in postmenopausal macaques. The clinical implication of this finding may be a greater risk for development of breast neoplasms in women receiving combined hormone replacement therapy. (Am J Obstet Gynecol 1996;174:93-100.)

**Key words:** *Macaca fascicularis,* hormone replacement therapy, mammary gland, steroid receptors, proliferation

Postmenopausal estrogen replacement has been shown to have major beneficial effects in the prevention of coronary heart disease and osteoporosis.[1] Unfortunately, the public health benefits of hormone replacement therapy have not been realized, largely because of the fear of cancer. A recent report indicates that among women in the United States concern over the risk of breast cancer is the greatest deterrent for the use of hormone replacement therapy.[2] This concern has some basis in the results of recent epidemiologic studies that suggest an increased risk of breast cancer in long-term current users of hormone replacement therapy.[1, 3, 4] The mechanistic basis for this increased risk is unknown. The mitogenic effects of estrogens on both breast and endometrial tissue are well recognized, as are the beneficial effects of progestogens on endometrial cell proliferation and cancer risk. The great controversy concerns the action of progestogens on breast tissue, where the literature offers a number of conflicting results both in vitro[5-7] and in vivo.[8-11] The assumption that breast and uterus are regulated similarly leads to the conclusion that the combined hormone replacement therapy designed to decrease the risk of endometrial cancer (i.e., estrogen plus a progestogen) is also appropriate for breast. A recent metaanalysis of studies including women treated with estrogen plus a progestogen did not show a protective effect of progestogen use.[3]

Rhesus and cynomolgus macaques are similar to women in many aspects of reproductive physiologic and anatomic features. Macaques have a distinct menarche and menopause, at about the ages of 3 and 20 years, respectively. They have a 28-day menstrual cycle, with a

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*Supported by grant No. DAMD17-94-J-4201 from the United States Army Medical Research Acquisition Activity, Fort Detrick, Maryland; grants No. HL-38964 and HL-45666 from the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland; the Swedish Medical Research Council (project No. 5982); the Karolinska Institute Research Funds; and the Swedish Cancer Society.*

*Received for publication December 7, 1994; revised April 28, 1995; accepted May 4, 1995.*

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*0002-9378/96 $5.00 + 0 6/1/66057*

**Table I.** Numbers of animals with atrophic or hyperplastic mammary glands by treatment

| *Treatment* | *Atrophy* | *Hyperplasia* | *Equivocal or not done* |
|---|---|---|---|
| Control ($n = 25$) | 23 (92%) | 0 | 2 (8%) |
| CEE ($n = 22$) | 11 (50%) | 9 (41%) | 2 (9%) |
| CEE plus MPA ($n = 21$) | 2 (9%) | 18 (86%) | 1 (5%) |

Numbers of animals with hyperplasia are significantly higher in conjugated equine estrogens plus medroxyprogesterone acetate group ($p = 0.0065$). *CEE*, Conjugated equine estrogens; *MPA*, medroxyprogesterone acetate.

hormonal profile similar to that of women.[12] Their endometrial responses to endogenous and exogenous hormones parallel those of women.[13] Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar.[14] Human and macaque mammary glands display the same cytokeratin types.[15] Mammary neoplasms are uncommon in macaques.[16] This is the first large study of the mammary responses of macaques to long-term hormone replacement therapy.

## Methods

**Animals.** The subjects of this study were 68 feral adult female cynomolgus monkeys *(Macaca fascicularis)* imported from Indonesia (Charles River Primates, Port Washington, N.Y.). The animals were part of an atherosclerosis-osteoporosis prevention trial, the results of which will be published elsewhere. They ranged in age from 5 to 13 years, as estimated from dentition, and were not pregnant. Animals were housed in social groups of four to eight monkeys each in a facility accredited by the American Association for Laboratory Animal Care. Experimental protocols were approved by the institutional Animal Care and Use Committee. Bilateral ovariectomies were done on all animals before the atherosclerosis induction period began.

**Diets and drug dosing.** The hormones were administered twice daily in a moderately atherogenic diet (40% of kilocalories from fat, 0.2 mg of cholesterol per kilocalorie). Monkeys were fed approximately 120 calories/kg per day. For 8 months of the 30-month treatment period groups receiving conjugated equine estrogens were given 7.2 μg of conjugated equine estrogens (Premarin, Wyeth-Ayerst, Radnor, Pa.) per monkey per day. For the remaining 22 months the dose was approximately 166 μg per monkey per day to be equivalent to women receiving 0.625 mg per day. Throughout the 30-month treatment phase the group receiving conjugated equine estrogens plus medroxyprogesterone acetate were given approximately 650 μg per monkey per day of medroxyprogesterone acetate (Cycrin, Wyeth-Ayerst), to be equivalent to a woman's dose of 2.5 mg per day. Drug doses were computed as (Human dose)/(1800 kilocalories per woman per day) = Dose per calorie of diet.

**Serum hormone measurements.** Before treatment measurements were made of estradiol and progesterone to confirm completeness of ovariectomy; estradiol and medroxyprogesterone acetate were measured during the treatment phase. Samples were taken 4 hours after feeding and dosing. Medroxyprogesterone acetate was measured by radioimmunoassay. Estradiol-17β was measured by a modification of a commercial kit (Diagnostic Products, Los Angeles). All hormone measurements were carried out at the Comparative Endocrinology Laboratory of the Yerkes Regional Primate Center of Emory University (Atlanta) by Dr. Mark Wilson.

**Tissue collection.** Mammary glands were collected at the end of the 30-month treatment phase, when all monkeys were killed and necropsies were performed. Samples were taken in the sagittal plane through the nipple and included a 2 to 3 cm segment of skin and gland. Tissues were fixed in 4% buffered paraformaldehyde for 24 hours and stored in 70% ethanol at 4° C. Tissues were then trimmed to 3 mm in thickness, embedded in paraffin, and sectioned at 5 μm for immunostaining.

**Histopathologic study.** Mammary gland slides were subjectively classified as atrophic, hyperplastic, or neither. The treatment group of each animal was obscured during the procedure to prevent observer bias. Hyperplasia, atypia, cystic lesions, and the presence of intraluminal protein or intraepithelial fat globules mimicking secretory activity were noted. Lesions were independently graded as none, mild, moderate, or severe.

**Morphometry and stereologic study.** Mammary gland thickness was measured as greatest thickness perpendicular to the skin from histologic sections. Measurements were made with an ocular micrometer at a magnification of ×20. An image analysis system (Bioquant, R and M Biometrics, Nashville, Tenn.) was used to measure mean nuclear area and nuclear roundness factor in 10 randomly selected cells each from alveoli, terminal ducts, and major ducts at a magnification of ×400. Nuclear roundness factor is defined as $4\pi(\text{area})/\text{perimeter}^2$. Estimates of the relative proportions of tissue components in the mammary gland were made by point counting.[17] A 10 × 10 grid was superimposed on the section, and intercept points over features of interest were counted to determine the percentage of gland occupied by epithelium, connective tissue, and fat. Numbers of points intercepting each lobule were also recorded as a relative indicator of lobular size. For each section measured, 10 lobules were assessed, requiring an average of 4.6 microscopic fields at a magnification of ×20.

**Sex steroid receptors and proliferation.** Staining procedures were done on fixed, paraffin-embedded tissues.

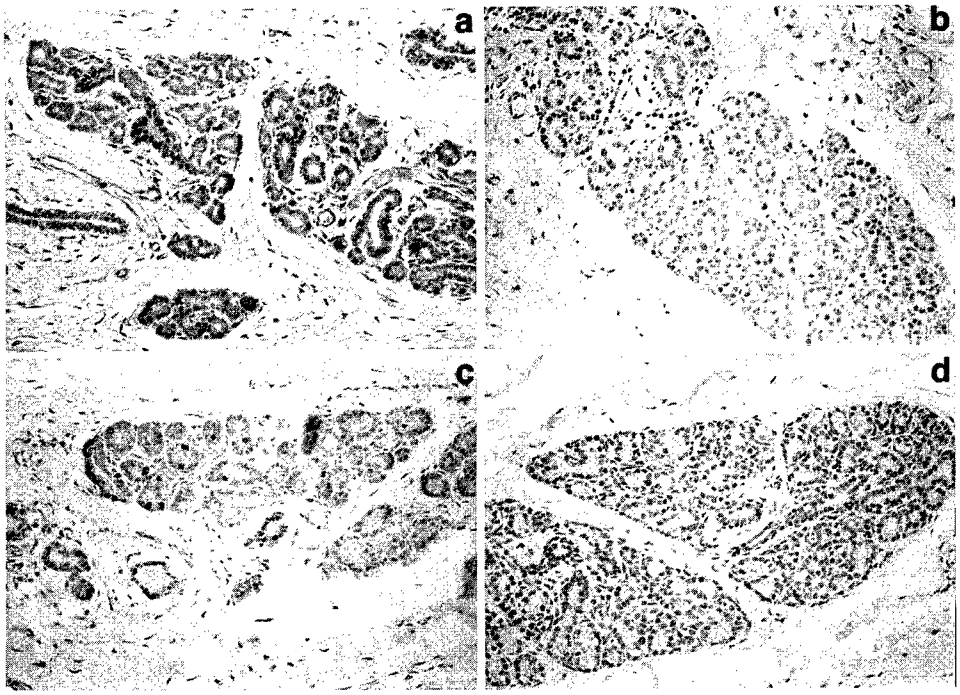


**Fig. 1.** Typical estrogen receptor staining (**a** and **b**) and progesterone receptor staining (**c** and **d**) in control animals (**a** and **c**) and in animals from conjugated equine estrogens plus medroxyprogesterone acetate group (**b** and **d**). Positively stained cells appear *black*. Loss of receptor staining is accompanied by increase in proportion of epithelial tissue.

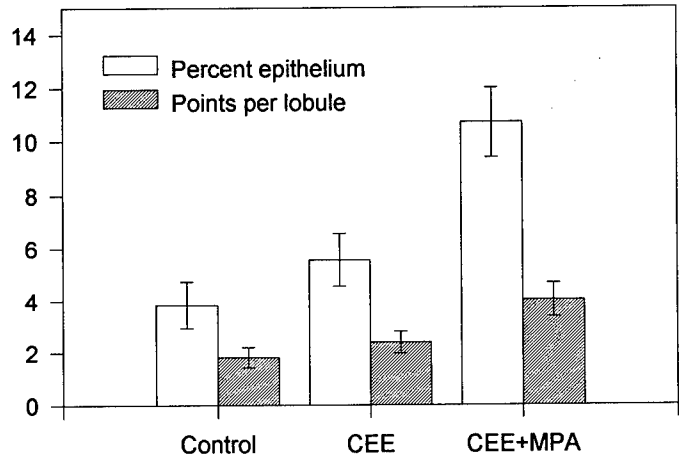


**Fig. 2.** Point-counting measurements of percentage ± SEM of epithelium relative to stroma and number of points counted per lobule in mammary gland of macaques. Both measures indicate glandular hyperplasia in conjugated equine estrogens *(CEE)* plus medroxyprogesterone acetate *(MPA)* group. For percent epithelium, the conjugated equine estrogens plus medroxyprogesterone acetate group differs from the conjugated equine estrogens group ($p < 0.05$) and from controls ($p < 0.0001$). For points per lobule both conjugated equine estrogens–treated and conjugated equine estrogens plus medroxyprogesterone acetate–treated animals differed from controls ($p < 0.05$ and $p = 0.0007$, respectively) but did not differ from each other.

The basic staining procedure is an avidin-biotin-peroxidase method for antigen retrieval from paraffin-embedded tissue.[18] The estrogen receptor and progesterone receptor analyses were performed with reagents supplied by Dako Laboratories (Carpinteria, Calif.) and Immunotech Laboratories (Marseille, France), respectively. To assess proliferation, we used the newly introduced KI-67 MIB-1 monoclonal antibody (Immunotech), which gives an immunostaining identical to Ki-67 antibody and which can be used on paraffin-embedded tissue sections.[18]

**Quantification of immunohistochemical staining.** Immunostained cells were quantified by cell counting in

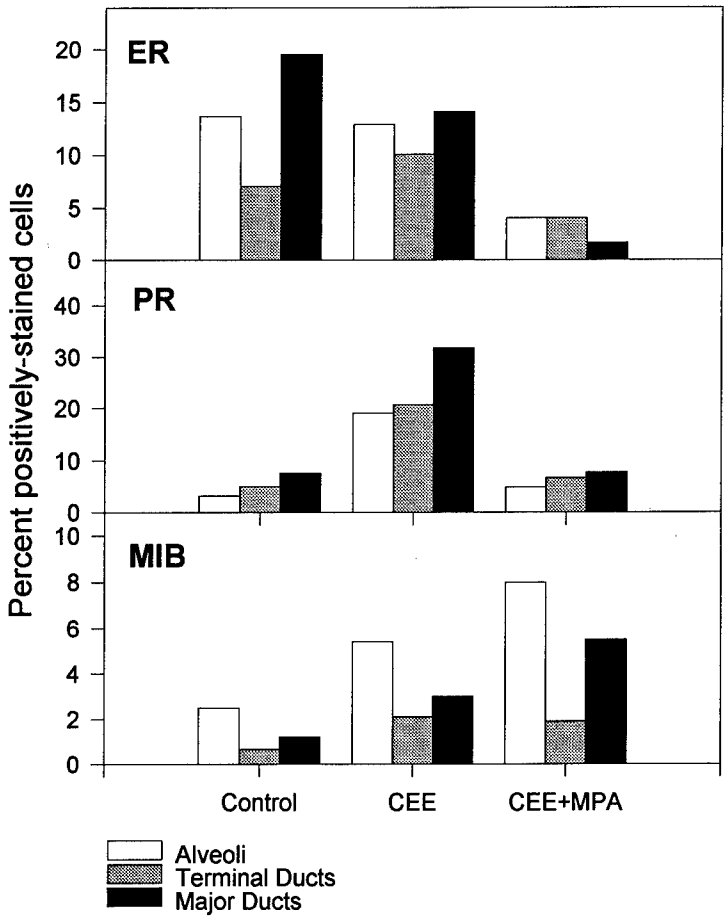


**Fig. 3.** Immunostaining of mammary epithelial cells. MIB-1 labeling is increased in both treatment groups, most notably in conjugated equine estrogens *(CEE)* plus medroxyprogesterone acetate *(MPA)* group. Estrogen receptor *(ER)* immunostaining is decreased in conjugated equine estrogens *(CEE)* plus medroxyprogesterone acetate *(MPA)* group. Progesterone receptor *(PR)* immunostaining is significantly increased only in group given conjugated equine estrogens alone (Tables II and III).

sections by an observer blinded to treatments. Epithelial cells lining the alveoli and the terminal and major ducts were considered separately to assess regional differences. Cell nuclei were identified as unlabeled (0) or weakly (+), moderately (++), or intensely (+++) labeled. At least 100 cells per slide were counted at three different sites for each combination of animal, tissue site, and stain type. Terminal ducts could not be identified in some cases.

**Statistical methods.** Statistical analysis was performed with the Mann-Whitney *U* test with Bonferroni corrections for multiple comparisons, the Kruskal-Wallis test, $\chi^2$ test, and Spearman's rank correlation test.

## Results

**Hormone measurements.** Plasma estradiol concentrations (mean ± SEM) were 5.0 ± 0.7, 167.1 ± 9.9, and 160.9 ± 13.9 pg/ml for controls, conjugated equine estrogens, and conjugated equine estrogens plus medroxyprogesterone acetate groups, respectively ($p <$ 0.0001 between control and treatment groups). The corresponding medroxyprogesterone acetate concentrations were 35.9 ± 6.1, 24.7 ± 3.8, and 116.2 ± 5.2 pg/ml ($p < 0.0001$ between untreated and medroxyprogesterone acetate–treated groups). Medroxyprogesterone acetate measurements for animals not given this drug were not significantly different from background.

**Subjective evaluation of mammary morphologic features.** Mammary gland atrophy was seen in nearly all control animals. Animals given conjugated equine estrogens alone had lobular atrophy or hyperplasia with equal frequency. Eighty-six percent of animals given conjugated equine estrogens plus medroxyprogesterone acetate had mammary hyperplasia, defined as greater mammary gland development than that seen in a normally cycling premenopausal macaque (Table I). Features similar to secretory differentiation were not related to treatment.

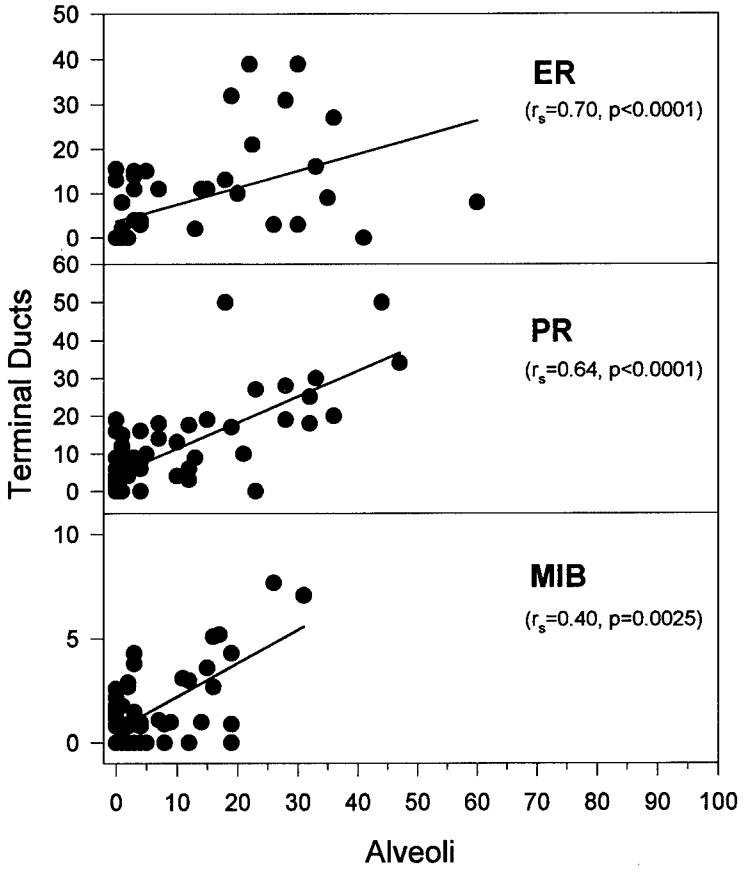


**Fig. 4.** Regression plots of correlations between immunostaining in alveoli and terminal ducts. Correlations are highly significant. *ER*, Estrogen receptor; *PR*, progesterone receptor.

**Morphometric and stereologic study.** Mammary gland thickness was significantly affected by treatment; the mean thickness (micrometers) ± SD was 264 ± 153 for controls, 396 ± 211 for animals given conjugated equine estrogens, and 444 ± 249 for animals given conjugated equine estrogens plus medroxyprogesterone acetate. Controls differed from both treated groups at $p < 0.05$ and from the conjugated equine estrogens plus medroxyprogesterone acetate group at $p < 0.01$. The percentage of mammary gland occupied by glandular tissue was increased in both treated groups, most markedly in animals given conjugated equine estrogens plus medroxyprogesterone acetate. Relative lobular size (expressed as points per lobule) was increased in animals given conjugated equine estrogens, and more so in animals receiving conjugated equine estrogens plus medroxyprogesterone acetate (Figs. 1 and 2).

Nuclear area was slightly increased in both hormonal treatment groups in the case of alveoli and terminal ducts. Nuclear roundness factor was slightly lower at all sites in the conjugated equine estrogens plus medroxyprogesterone acetate group. Nuclear changes did not reach statistical significance.

**Immunostaining for estrogen and progesterone receptors and proliferating cells.** Table II illustrates the percentage of all receptor-positive cells for the different groups. The percentage of estrogen receptor–positive cells was decreased in both treatment groups, most markedly in the conjugated equine estrogens plus medroxyprogesterone acetate group (Fig. 3). The percentage with positive staining for estrogen receptor and those intensely labeled (+++) was higher in the control and conjugated equine estrogens groups than in the conjugated equine estrogens plus medroxyprogesterone acetate group. Significant differences were found between the total number of receptor-positive cells in the conjugated equine estrogens and conjugated equine estrogens plus medroxyprogesterone acetate groups for alveoli, terminal ducts, and major ducts. There were highly significant correlations between the percentages of estrogen receptor–positive cells of the alveoli and ducts ($r_s = 0.69$, $p < 0.0001$), alveoli and terminal ducts ($r_s = 0.70$, $p < 0.0001$), and between terminal ducts and ducts ($r_s = 0.64$, $p < 0.0001$) (Fig. 4).

The percentage of progesterone receptor–positive cells was higher in the conjugated equine estrogens group than in both the control and conjugated equine estro-

**Table II.** Mean percentage of receptor-positive breast epithelial cells from cynomolgus macaques

| | *ER (mean, range)* | *PR (mean, range)* |
|---|---|---|
| Alveoli | | |
| Group A, control | 13.7 (0-63) ($n = 24$) | 3.2 (0-28) ($n = 25$) |
| Group B, CEE | 12.9 (0-60) ($n = 22$) | 19 (0-47) |
| Group C, CEE plus MPA | 4.1 (0-28) ($n = 19$) | 4.8 (0-32) |
| Significant differences | *B* vs *C*, $p = 0.014$ | *A* vs *B*, $p = 0.0003$<br>*A* vs *C*, $p = 0.034$<br>*B* vs *C*, $p = 0.0006$ |
| Terminal ducts | | |
| Group A, control | 7.1 (0-39) ($n = 18$) | 5.0 (0-27) ($n = 21$) |
| Group B, CEE | 10.1 (0-39) ($n = 19$) | 20.7 (6-50) |
| Group C, CEE plus MPA | 4.1 (0-31) ($n = 18$) | 6.6 (0-25) ($n = 19$) |
| Significant differences | *B* vs *C*, $p = 0.035$ | *A* vs *B*, $p = 0.0003$<br>*B* vs *C*, $p = 0.0003$ |
| Major ducts | | |
| Group A, control | 19.5 (0-76) ($n = 26$) | 7.5 (0-43) |
| Group B, CEE | 14.1 (0-52) ($n = 22$) | 31.8 (0-56) |
| Group C, CEE plus MPA | 1.7 (0-18) ($n = 19$) | 7.7 (0-33) |
| Significant differences | *B* vs *C*, $p = 0.003$<br>*A* vs *C*, $p = 0.007$ | *A* vs *B*, $p = 0.0003$<br>*B* vs *C*, $p = 0.0003$ |

Number of evaluable specimens is indicated. *ER*, Estrogen receptor; *PR*, progesterone receptor; *CEE*, conjugated equine estrogens; *MPA*, medroxyprogesterone acetate.

**Table III.** Mean percentage of cells with MIB-1 staining in breast epithelial cells of cynomolgus macaques

| | *Staining intensity* | |
|---|---|---|
| | *All stained cells (mean, range)* | *+++ only (mean, range)* |
| Alveoli | | |
| Group A, control ($n = 25$) | 2.5 (0-19) | 0.08 (0-2) |
| Group B, CEE ($n = 22$) | 5.4 (0-26) | 0.14 (0-1) |
| Group C, CEE plus MPA ($n = 19$) | 8.0 (0-31) | 0.84 (0-7) |
| Significant differences | *A* vs *C*, $p = 0.016$ | *A* vs *C*, $p = 0.009$ |
| Terminal ducts | | |
| Group A, control ($n = 20$) | 0.6 (0-3) | 0 |
| Group B, CEE ($n = 22$) | 2.1 (0-8) | 0.04 (0-1) |
| Group C, CEE plus MPA ($n = 19$) | 1.9 (0-7) | 0.13 (0-2) |
| Significant differences | — | — |
| Major ducts | | |
| Group A, control ($n = 26$) | 1.2 (0-10) | 0 |
| Group B, CEE ($n = 22$) | 3.0 (0-14) | 0.32 (0-2) |
| Group C, CEE plus MPA ($n = 19$) | 5.5 (0-28) | 0.84 (0-9) |
| Significant differences | *A* vs *C*, $p = 0.017$ | *A* vs *B*, $p = 0.015$<br>*A* vs *C*, $p = 0.046$ |

Number of evaluable specimens is indicated. *CEE*, Conjugated equine estrogens; *MPA*, medroxyprogesterone acetate.

gens plus medroxyprogesterone acetate groups in all three histologic sites (Fig. 3). In all animals there were highly significant correlations between the percentages of progesterone receptor–positive cells of the alveoli and major ducts ($r_s = 0.76$, $p < 0.0001$), alveoli and terminal ducts ($r_s = 0.64$, $p < 0.0001$), and between terminal ducts and major ducts ($r_s = 0.64$, $p < 0.0001$) (Fig. 4).

Table III shows the proportion of MIB-1–positive cells for the different histologic cell types and treatments. The treated groups in general had a larger proportion of proliferating cells than controls, with the highest proportion in the conjugated equine estrogens plus medroxyprogesterone acetate group. Significantly higher values were seen in the conjugated equine estrogens plus medroxyprogesterone acetate group relative to untreated controls in alveoli and major ducts, and there were also significantly higher values for the conjugated equine estrogens group relative to controls for intensely labeled cells in the major ducts. There was a strongly significant correlation between percentages of positive cells in alveoli and terminal ducts ($r_s = 0.40$, $p = 0.0025$, Fig. 4) and between alveoli and major ducts ($r_s = 0.32$,

$p < 0.001$) but not between terminal ducts and major ducts.

Regarding serum concentrations of hormones, the following correlations had an $r_s$ value of 0.25 to 0.5; all had a $p$ value $\leq 0.05$. Higher serum concentrations of medroxyprogesterone acetate were positively correlated with MIB-1 labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. Negative correlations were seen with estrogen receptor and progesterone receptor labeling. Higher serum concentrations of estradiol were positively correlated with MIB-1 labeling, progesterone receptor labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. A negative correlation was seen between serum estradiol and estrogen receptor labeling.

When correlation testing was done for serum hormone concentrations within treatment groups, only medroxyprogesterone acetate concentrations were positively correlated with any immunostaining parameter (alveolar cells with positive MIB-1 staining, $r_s = 0.49$, $p = 0.035$, and strong alveolar staining, $r_s = 0.52$, $p = 0.024$).

## Comment

Hormonal regulation of the normal breast and hormonal risk factors for the development of breast cancer remain a subject of controversy. In the normal menstrual cycle of women proliferation occurs primarily during the luteal phase of the cycle, indicating that breast does not respond to the same proliferative stimuli as the endometrium.[19] Human and nonhuman primate mammary glands have many similarities in anatomic features, hormonal regulation,[14] and cytokeratin immunophenotype[15] that are not shared by the commonly used laboratory rodents. We believe that the macaque model offers a unique opportunity for study of mammary gland regulation because it enables evaluation of the effects of long-term hormone replacement therapy on various locations in the breast of healthy subjects.

Morphometric and stereologic evaluation of tissues in this study clearly indicate a mammotropic effect of conjugated equine estrogens plus medroxyprogesterone acetate, which appears to exceed that of conjugated equine estrogens alone. This study shows a down-regulation of both estrogen and progesterone receptors in breast epithelium during combined treatment, similar to that of the endometrium. However, there was a significantly greater gland thickness and percentage of epithelial tissue in animals receiving combined therapy versus those receiving conjugated equine estrogens only. Also in contrast to the endometrium, there is a clear trend of increased proliferative activity of the breast epithelium on combined therapy. This is in line with studies that suggest an increased breast cancer risk associated with combined estrogen-progestin therapy.[10] It is, however, important to note that there is no statistical difference between Ki-67 (MIB-1) labeling in estrogen replacement therapy and combined hormone replacement therapy. The tendency for increased proliferation in the combined therapy group was accompanied by decreased proportions of progesterone receptor–positive cells. Previously our group[20] and others[4] have found a sustained progesterone receptor level under the influence of progesterone during the luteal phase of the menstrual cycle. These findings indicate that estrogen receptors in breast are down-regulated by progesterone, as in uterus,[21] but that progesterone receptor positivity in breast does not change during the course of normal cycles. Progesterone is a well-known stimulator of lobulo-alveolar development. Apparently there are many differences between cyclic progesterone and continuous medroxyprogesterone acetate. We have recently shown that progesterone increases the intratissue formation of estrone from estrone sulfate, whereas norethisterone acetate in combined oral contraceptives does not because of differences in sulfatase activity induced by these two compounds.[22]

The basis of risk associated with hormonal therapies may lie in regulation of cell proliferation. Within populations of cells in vitro and in vivo, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well.[4] The murine monoclonal antibody Ki-67 reacts with a human deoxyribonucleic acid binding protein that is present in proliferating cells but absent in quiescent cells. A detailed cell-cycle analysis showed that the Ki-67 antigen is expressed in $G_1$, S, $G_2$, and mitosis (with maximum levels during $G_2$ and M phases) but not in $G_0$, and by use of this antibody an exact determination of the growth fraction of a given human cell population, regardless of whether it is normal or malignant, has been possible.[23]

The proliferation of breast cells in vitro has mostly been found to be stimulated by estrogens and inhibited by progestogens. However, in these experiments cultured epithelial cells are deprived of their normal complement of blood vessels, adipose tissue, stroma, and myoepithelial cells. These surrounding cells exert considerable paracrine and hormonal influence in vivo. Experiments with human tissue so far have been tritiated thymidine labeling and mitosis analyses on tissue sections from reduction mammoplasties or from "normal" breast tissue near a benign or malignant lesion. Most of these studies have demonstrated greater proliferation during the luteal phase.[4]

Clarke and Sutherland[24] postulated that progestogens could activate the cell cycle for one turn and that prolonged stimulation would turn it off. Our study seems to contradict this hypothesis because 2 years of prolonged continuous combined therapy significantly enhances breast cell proliferation. There is one report of a direct stimulatory effect of 19-nor steroids on estrogen receptor–positive breast cancer cells by the estrogen re-

ceptor.[25] The synthetic progestogen R 5020 stimulates insulin-mediated breast cancer cell proliferation by increasing insulin receptors and insulin receptor messenger ribonucleic acid content; this may be another mechanism of action on normal breast epithelial cells.[7] Further studies from our group will evaluate this hypothesis and also sex steroid receptor variation, breast cell proliferation, and growth factors of both cycling and oral contraceptive–treated healthy volunteers and cynomolgus monkeys.

We thank Torsten Hagerström, Berit Stäbi, Britt Kotander, Birgitta Bystrom, Audrey Kelleman, and Brian McCollough.

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# REGIONAL DISTRIBUTION OF PROLIFERATING CELLS AND HORMONE RECEPTORS IN THE MAMMARY GLAND OF SURGICALLY POSTMENOPAUSAL MACAQUES

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This work was supported in part by Grants HL- 38964 and HL 45666, National Heart Lung and Blood Institute, National Institutes of Health Bethesda, MD and by the Swedish Medical Research Council (Project No. 5982), the Karolinska Institute Research Funds, and the Swedish Cancer Society.

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## SUMMARY

Objectives: To define the relative proliferative response and hormone receptor status in 10 sites in the mammary gland of surgically postmenopausal cynomolgus macaques given hormone replacement therapy.

Study design: Surgically postmenopausal cynomolgus macaques were given either no treatment (n=4), conjugated equine estrogens (CEE, n=4) or combined therapy with CEE and medroxyprogesterone acetate( CEE+MPA, n=4). Drugs were administrated in the diet, at doses equivalent on a caloric basis to 0.625 mg/ woman /day for CEE and 2.5mg/ woman /day for MPA. Immunostaining of mammary sections was done for estrogen receptor (ER), progesterone receptor (PR, and the proliferation marker Ki-67 MIB-1 (MIB). Comparisons were made between central and peripheral gland, by quadrant, left vs. right, and with respect to distance from the nipple within each quadrant.

Results: There were no significant differences in hormone receptor or MIB expression in different sites within the gland.

Conclusion: In the surgically postmenopausal, hormone treated macaque, regional differences in ER, PR and MIB staining are not apparent. The assumption of homogeneity throughout the gland makes aspiration cytology and multiple biopsy studies feasible in this species.


Key words: *Macaca fascicularis* , mammary gland, regional differencies, steroid receptors, proliferation.

## INTRODUCTION

Breast cancer is the dominant malignancy among women in Western countries. The understanding of normal breast physiology and its responses to endogenous and exogenous sex hormone stimuli is vital in this context. Many studies have indicated that the tissue of the breast is inhomogenous during early development and in puberty (1, 2). Regional differences in tumor incidence within the breast tissue are well documented(3- 5), and about 50% of breast cancers are located to the upper lateral quadrant. Also cancer tends to be somewhat more frequent in the left breast than in the right (5). Holland et al reported in 1985 that multiple tumor foci within the breast are regionally distributed or clustered (6). Differences with respect to estrogen receptor expression between lobules in a single breast were found in premenopausal women subject to bilateral mastectomy for benign conditions (7). Experimental evidence suggests that not all cells in the breast are responsive to steroid hormones. While in another target organ like the endometrium during the menstrual cycle virtually all cells proliferate on low estradiol levels, in the breast the number of proliferating cells is only about 3-5% (8-10). How these cells might be distributed in the breast remains to be answered.

In studies to explore normal breast physiology and response to sex steroids, biopsy or fine needle aspirate samples are often used. Therefore it is necessary to understand how these samples may be biased by random or predictable variation in cellular proliferation, hyperplasia, dysplasia or neoplasia. Such studies for obvious reasons are difficult to perform on normal breast tissue from healthy women. We recently evaluated the effects of long term hormonal replacement therapy on the mammary gland of surgically postmenopausal macaques (11). In the present study an immunohistochemical analysis of the regional distribution of estrogen

receptor (ER), progesterone receptor (PR) and the proliferation marker Ki-67 MIB-1 (MIB), in the macaque breast was performed.

Features of the macaque model

Rhesus and cynomolgus macaques are similar to women in many aspects of reproductive physiology and anatomy. Macaques have a distinct menarche and menopause, at about 3 and 20 years of age, respectively. They have a 28-day menstrual cycle, with a hormonal profile similar to that of women (12). Their endometrial responses to endogenous and exogenous hormones parallel those of women (13). Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar (14- 16). There are functional and structural differences between species that support the use of the primate model, rather than other animal species. These include mammary proliferative and tumor promoting responses to prolactin in rodents and other nonprimate species, (17,18), which are not profound in macaques (19, 20) or humans (21). Macaque and human mammary epithelial cells also share a characteristic cytokeratin phenotype, which differs from that of rodents (22), reflecting the phylogenetic closeness of the species. Macaques thus should provide a better model than rodents for the assessment of proliferation and hormonal regulation of the human mammary gland.

## METHODS

Animals

The subjects of this study were 12 feral adult female cynomolgus monkeys (*Macaca fascicularis*) imported from Indonesia (Charles River Primates, Port Washington, NY). The animals were part of an atherosclerosis/osteoporosis prevention trial, the results of which are published elsewhere (11). They ranged in age from 5 to 13 years as estimated from dentition and were not

pregnant. Animals were housed in social groups of 4-8 monkeys each, in an AALAC- accredited facility; experimental protocols were approved by the institutional Animal Care and Use committee. Bilateral ovariectomies were done on all animals prior to treatment.

Diets/Drug Dosing

Hormones were administered twice daily in the diet. Monkeys were fed approximately 120 Calories per kg of body weight per day. Details of diet composition and hormonal treatment are published elsewhere (11). Four monkeys were given conjugated equine estrogens (CEE) (Premarin®, Wyeth-Ayerst, Radnor, PA) approximately 166 μg per monkey per day to be equivalent to women receiving 0.625 mg per day. In four monkeys, medroxyprogesterone acetate (MPA) (Cycrin®, Wyeth-Ayerst) approximately 650 μg per monkey per day, equivalent to a woman's dose of 2.5 mg per day, was added to the estrogen and four monkeys received no hormonal treatment.

Tissue collection

Mammary gland was collected at the end of the 30 months treatment phase, when all monkeys were euthanized and necropsied. The distribution of samples collected is illustrated in Figure 1. A single sample, (sample 1) was taken from the center of the breast, including cross-section of the nipple. Two samples were taken from each quadrant of the right breast (samples 2-9) including tissue from 1-2 and 2-3 cm from the nipple. A single sample from the left breast was examined (sample 10), which corresponded in location to sample 4. Tissues were fixed in 4% paraformaldehyde at 4°C. The fixed tissue was removed from paraformaldehyde after 24 hours, and stored in 70% ethanol at 4°C. Fixed tissues were trimmed to 3 mm in thickness, embedded

in paraffin using standard histologic procedures, and sectioned at 5 μm for immunostaining.

Sex steroid receptors

Staining procedures were done on fixed, paraffin-embedded tissues. The basic staining procedure uses an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin-embedded tissue (11, 23). The estrogen receptor and progesterone receptor analyses were performed with reagents supplied by Dako laboratories (Dako Corporation, Carpinteria, CA, USA), and Immunotech laboratories (Immunotech, Marseille, France), respectively.

Assessment of proliferation

We used the newly introduced KI-67 MIB-1 monoclonal antibody (Immunotech, Marseille, France) that gives an immunostaining identical to the Ki-67 antibody and which can be used on paraffin embedded tissue sections (11, 24). As for the receptor analysis, the MIB basic staining procedure uses an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin embedded tissue.

Quantification of immunohistochemical staining

Immunostained cells were quantified by cell counting in sections, by an observer blinded to treatments. Epithelial cells lining the alveoli/terminal ducts and major ducts were considered separately. Labeled cell nuclei were identified as unlabeled (0), weakly labeled (1+), moderately labeled (2+), or intensely labeled (3+). At least 100 cells per slide were counted at 3 different sites for each combination of animal, tissue site and stain type.

Statistical methods.

Statistical analysis was performed using the Kruskal -Wallis test and Spearman's rank correlation test.

## RESULTS

The median and range for the percentage of all cells positive for estrogen and progesterone receptor by site is illustrated in Table 1. There were no differences between the 10 sites sampled.The distal part of the lower median quadrant (site 7 ) in most cases contained no evaluable tissue for either alveoli or ducts. Fig. 2 shows the median and range values for the receptors by quadrant for alveoli and ducts. There were no differences between any of the quadrants. The median values of the outer and inner (proximal and distal) parts of the quadrants also showed no difference. Samples taken from the corresponding lower lateral quadrant in the left (site 10) and right (site 4) breast were not significantly different with respect to any stain.

The MIB staining for the 10 sampled regions is illustrated in Table 2. No site differed statistically from any of the other. Proliferative activity by quadrant and by distance from the nipple is illustrated in Figure 3. Comparison between quadrants for both alveoli and ducts revealed no difference. Also, when comparing the median MIB value of the outer and inner parts of the quadrants, no differences were found.

## COMMENT

The findings reported here indicate that there is no difference in epithelial proliferation or sex steroid receptor expression between different regions of the macaque breast. Regional differences in tumor incidence in women could be due to local differences in proliferative activity (6, 7), but have also been attributed simply to the relative proportion of mammary tissue at risk in each quadrant (4, 5). Our findings support the latter hypothesis in the macaque model. This explanation implies that there is no intrinsic difference in the regulation of mammary tissue in various quadrants of the breast.

There is considerable work indicating that there are differences in enzymatic (aromatase, sulfatase, and dehydrogenase) activity in tumor- bearing regions of the breast (25- 30). There are two possible explanations for these findings: Either tumors secrete factors capable of enhancing enzymatic activity, or conversely increased enzymatic activity in a particular quadrant results in a local environment which encourages tumor development at that site. The present data indicate that these changes are likely to be secondary to tumor development rather than being pre-existing differences. Clearly breast cancer cells can secrete growth- promoting factors which may stimulate hormone metabolism and proliferation in adjacent tissues (27, 29- 31). In agreement with our finding, Anderson et al found no difference in breast proliferation with respect to left and right breast (8).

The role of endogenous and exogenous hormones in the genesis of breast cancer is widely debated. The basis of risk associated with hormonal therapies may lie in the regulation of cell proliferation. Within populations of cells, *in vitro* and *in vivo*, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well. According to *in vivo* studies, only a minor fraction of normal cells are in a proliferative state during the normal cycle or during hormonal therapy (8, 10, 11, 32, 33); this was also the case in the present study in which median proliferation was 3- 16%, without any regional differences.

Human and non-human primate mammary gland have many similarities, in terms of anatomy, hormonal regulation and cytokeratin immunophenotype, that are not shared with commonly used laboratory rodents. We believe that the macaque model offers an unique opportunity for study of mammary gland regulation since it enables in situ evaluation of proliferation in quiescent and hormone treated breast from healthy subjects.

The present findings indicate that cytologic or biopsy based studies of the normal breast are unlikely to be biased by intrinsic regional differences in proliferation and receptor expression.

ACKNOWLEDGMENT

We thank Torsten Hägerström, Birgitta Byström, Britt Kotander, Audrey Kelleman, and Brian McCollough.

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**Captions to figures**

Fig. 1 Distribution of tissue samples collected from the breasts of surgically postmenopausal cynomolgus macaques.

Fig. 2 Percentage of estrogen and progeterone receptor positive cells by quadrant in alveoli and ducts of macaque breast. Values are median and range.

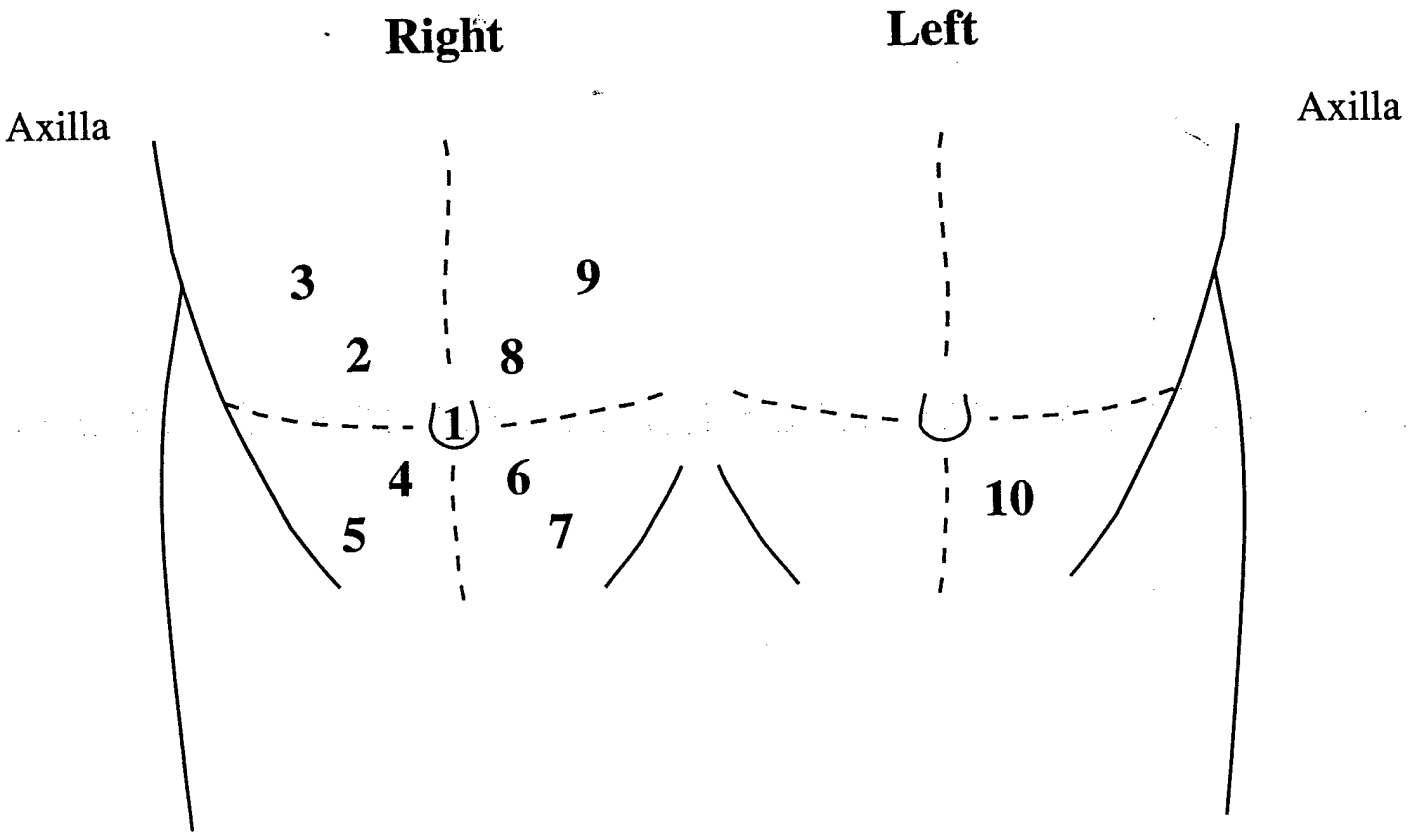
Fig. 3 Percentage of MIB positive cells by quadrant and by distance from the nipple in alveoli and ducts of macaque breast. Values are median and range.

**Table 1.** Median and range for the percentage of all cells positive for estrogen and progesterone receptor in alveoli and ducts in 10 sampled regions of macaque breasts.

| | **Alveoli** | | | | | | **Ducts** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ER | | | PR | | | ER | | | PR | | |
| Site | median | range | n | median | range | n | median | range | n | median | range | n |
| 1 | 36.5 | (0-77) | 12 | 13.5 | (0-49) | 12 | 27 | (0-44) | 12 | 20,5 | (1-42) | 12 |
| 2 | 40.5 | (1-79) | 12 | 24 | (0-87) | 11 | 37.5 | (0-62) | 12 | 21 | (0-86) | 11 |
| 3 | 36 | (1-66) | 12 | 19,5 | (1-81 | 12 | 28 | (0-63) | 12 | 15 | (0-79) | 11 |
| 4 | 44,5 | (15-67) | 12 | 21 | (7-90) | 11 | 33 | (3-68) | 12 | 36 | (16-88) | 11 |
| 5 | 34 | (8-76) | 11 | 21 | (5-93) | 9 | 33 | (2-66) | 10 | 50.5 | (0-96) | 8 |
| 6 | 29 | (7-81) | 11 | 26.5 | (0-56) | 10 | 20.5 | (2-54) | 10 | 18 | (0-51) | 9 |
| 7 | 31.5 | (17-82) | 4 | 12 | (12-12) | 1 | 16.5 | (2-31) | 2 | 73 | (73-73) | 1 |
| 8 | 30 | (20-81) | 10 | 21.5 | (0-83) | 10 | 29 | (2-48) | 9 | 30 | (8-92) | 9 |
| 9 | 54 | (12-77) | 8 | 11 | (1-65) | 7 | 29 | (0-39) | 5 | 4 | (0-65) | 6 |
| 10 | 35 | (10-70) | 11 | 22 | (0-67) | 11 | 22 | (6-68) | 11 | 26 | (0-76) | 10 |

**Table 2.** Median and range for the percentage of MIB-positive cells in alveoli and ducts in 10 sampled regions of macaque breasts.

| | **Alveoli** | | | **Ducts** | | |
|---|---|---|---|---|---|---|
| | MIB | | | MIB | | |
| Site | median | range | n | median | range | n |
| 1 | 6.5 | (0-39) | 12 | 0.5 | (0-15) | 12 |
| 2 | 19.5 | (0-39) | 12 | 2 | (0-15) | 12 |
| 3 | 14.5 | (0-50) | 12 | 2 | (1-10) | 12 |
| 4 | 23 | (0-38) | 9 | 3 | (0-6) | 10 |
| 5 | 19 | (1-50) | 9 | 2 | (0-15) | 9 |
| 6 | 8 | (0-41) | 11 | 1 | (0-4) | 7 |
| 7 | 26.5 | (14-39) | 2 | 4 | (4-4) | 1 |
| 8 | 12.5 | (2-45) | 10 | 1 | (0-7) | 8 |
| 9 | 7 | (0-41) | 9 | 1 | (0-8) | 9 |
| 10 | 10 | (1-47) | 11 | 2 | (0-12) | 11 |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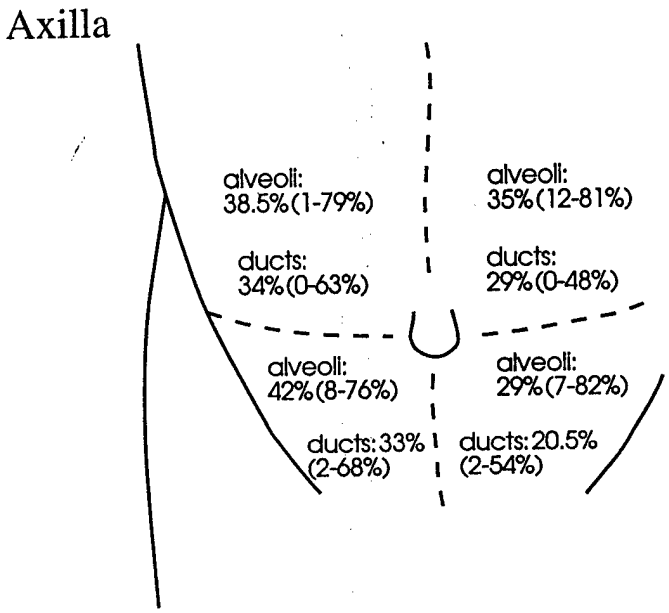
## **ER content** (right breast)

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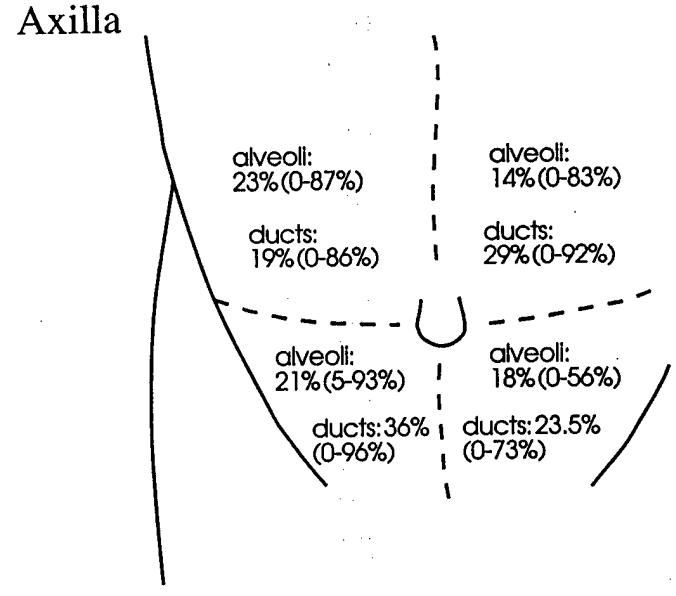
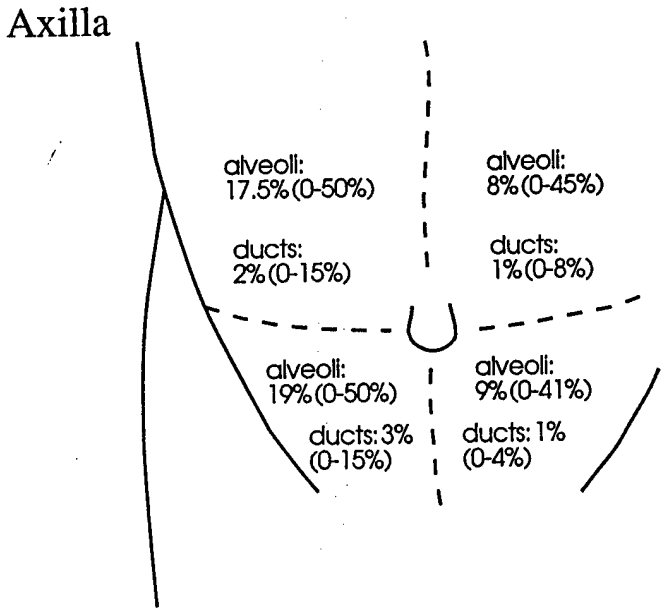
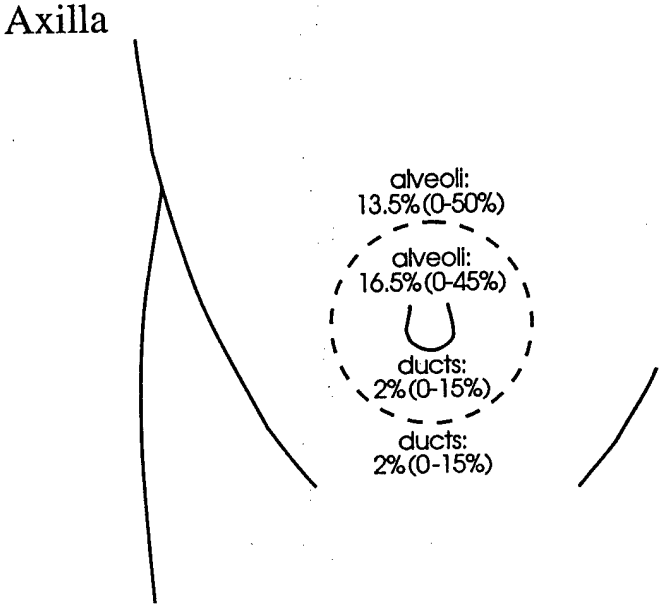


**MIB** (right breast)




**MIB** (right breast)



FERTILITY AND STERILITY
Vol. 65, No. 5, May 1996

*Printed on acid-free paper in U. S. A.*

# Effects of hormonal therapies and dietary soy phytoestrogens on vaginal cytology in surgically postmenopausal macaques*

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**Objective:** To evaluate the effects of conjugated equine estrogens, medroxyprogesterone acetate (MPA), conjugated equine estrogens combined with MPA, tamoxifen, and soybean estrogens on vaginal cytology in surgically postmenopausal cynomolgus macaques (*Macaca fascicularis*).
**Design:** Randomized long-term experimental trial.
**Setting:** Cytologic samples were taken from animals in two long-term randomized studies of the effects of hormonal and dietary effects on atherosclerosis.
**Patients:** Surgically postmenopausal cynomolgus macaques.
**Interventions:** Conjugated equine estrogens, MPA, conjugated equine estrogens combined with MPA, tamoxifen, and soybean estrogens were given via the diet, at doses scaled from those given to women.
**Main Outcome Measure:** Vaginal cytologic maturation index.
**Results:** Conjugated equine estrogens elicited a marked maturation effect, which was antagonized partially by the addition of MPA. Tamoxifen produced a lesser estrogenic response. The cytologic pattern in animals given soybean estrogens or MPA alone did not differ from that of controls.
**Conclusion:** Soybean estrogens at the doses given do not exert an estrogenic effect on the vagina of macaques. Conjugated equine estrogens are potent inducers of vaginal keratinization in this model; tamoxifen has a lesser effect. Medroxyprogesterone acetate partially antagonizes the effects of conjugated equine estrogens, and has no effect when given alone. The results support the possibility that soybean estrogens may be a "tissue-selective" estrogen with minimal effects on the reproductive tract. Fertil Steril 1996;65:1031–5

**Key Words:** Conjugated estrogens, tamoxifen, medroxyprogesterone acetate, isoflavones, phytoestrogens, vaginal cytology, *Macaca fascicularis*, soybeans

Estrogen replacement therapies provide beneficial effects for women in terms of heart disease and osteoporosis, but are associated with an increased risk of endometrial and breast neoplasia (1). This presents a therapeutic dilemma, which might be resolved by exploiting tissue selectivity among different estrogens or estrogen and progestin combinations. Presently, two agents with mixed estrogen agonist or antagonist properties are used widely by women. Soy foods, which are rich in estrogenic compounds (the phytoestrogens genistein and daidzein), are advocated widely in the lay literature as a substitute for estrogen replacement therapy (2). Tamoxifen is a commonly used breast cancer chemotherapeutic that also may be an effective chemopreventive agent in women at high risk of breast cancer (3). Because the exposed population for each of these agents is large, a better understanding of these potential selective estrogens is needed.

Received June 12, 1995; revised and accepted November 15, 1995.
* Supported in part by grants PO1 HL45666 and RO1 HL49085, National Heart, Lung and Blood Institute, Bethesda, Maryland; and DAMD17-94-J-4201, United States Army Medical Research Acquisition Activity, Fort Detrick, Maryland.
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Studies in humans and monkeys indicate that phytoestrogens (soybean estrogens) may have beneficial effects on cardiovascular risk factors (4, 5). Soy consumption also is associated with decreased breast and endometrial cancer rates, implying either a lack of estrogenicity or estrogen antagonism at these sites (6, 7). An equivocal estrogenic effect has been reported in vaginal smears of women given soy supplementation (8). Tamoxifen is known for its anticancer effects in breast, but produces an increased risk of high-grade endometrial cancer (3, 9). Tamoxifen also exerts a weak estrogenic effect on the vaginal epithelium of women (10). We believe that the multisystemic effects of hormonal therapies require an animal model in which multiple organ endpoints can be evaluated. The cynomolgus macaque model has proven useful in studies of the effects of endogenous and exogenous estrogens on cardiovascular risk factors, coronary artery atherosclerosis, vasomotor function (11), and osteoporosis (12), because it permits concurrent assessment of multiple tissue endpoints in individual animals. Cynomolgus macaques are similar to women in most aspects of their reproductive physiology (13, 14). Their endometrial and mammary responses to endogenous and exogenous hormones parallel those of women (15, 16). In these studies we compared the vaginal epithelial responses of surgically postmenopausal female macaques given tissue-selective estrogens (soybean estrogens or tamoxifen) with those of monkeys given more conventional hormonal therapies such as conjugated equine estrogens and conjugated equine estrogens combined with medroxyprogesterone acetate (MPA).

## MATERIALS AND METHODS

The subjects of this study were feral adult female cynomolgus monkeys (*Macaca fascicularis*) imported directly from Indonesia (Institut Pertanian Bogor, Bogor, Indonesia). The animals were subjects in studies of atherosclerosis and osteoporosis prevention, results of which will be published elsewhere. Animals were housed in social groups of four to eight monkeys each, in a facility accredited by the American Association for Laboratory Animal Care (Bethesda, MD). Experimental protocols were approved by the institutional Animal Care and Use Committee. Bilateral ovariectomies were done on all animals during 1991 (study 2) or 1994 (study 1), using ketamine hydrochloride (15 mg/kg) + butorphanol (0.025 mg/kg) for anesthesia.

Study 1: a subset of 40 macaques was sampled randomly from among 220 animals that had been randomized to either an ovariectomized control group (OVX) or one of two treatments, conjugated equine estrogens (Premarin; Wyeth-Ayerst, Princeton, NJ) or soybean estrogens administered as a soy protein isolate (SUPRO 670; Protein Technologies International, St. Louis, MO). Sampling was done 6 months after the beginning of treatments.

Study 2: One hundred sixteen macaques were randomized into five treatment groups: OVX, conjugated equine estrogens, conjugated equine estrogens combined with MPA, MPA, and tamoxifen. All animals were sampled 3 years after the beginning of treatments.

### Diets and Drug Dosing

Treatments and doses were chosen for their similarity to those that might be used in women. The hormones were administered twice daily in a moderately atherogenic diet (40% of calories from fat, 0.2 mg of cholesterol per kilocalorie). For study 1, the protein source for control and conjugated equine estrogens–containing diets was isoflavone-extracted soy protein. Monkeys were fed 120 kcal/kg of body weight per day. Drug doses were computed as the human dose divided by 1,800 kcal per woman per day to derive the dose per calorie of diet consumed (5). Therefore, doses arrived at by this means were scaled consistently and were adjusted for metabolic rate. The dose of conjugated equine estrogens in both studies was 166 $\mu$g per monkey per day, the monkey equivalent of a women receiving 0.625 mg/d. Groups receiving conjugated equine estrogens combined with MPA were given 650 $\mu$g per monkey per day of MPA (Cycrin; Wyeth-Ayerst), equivalent to a woman's dose of 2.5 mg/d. The group given tamoxifen received 4 mg, equivalent to a woman's dose of 20 mg/d. Soybean estrogens were given at a dose of approximately 26.6 mg free genistein per monkey per day; this is equivalent to a dose of 99.7 mg of genistein per woman per day and is the maximal soybean estrogens dose obtainable by large-scale processing methods.

### Cytologic Sampling

Vaginal cytologic samples were taken concurrent with blood collection for hormone analyses. Vaginal epithelial cells were collected from the anterior vagina of sedated animals (ketamine HCl, 15 mg/kg) using a moistened cotton swab. Cells were rolled onto a glass slide, were fixed immediately using a commercial aerosol fixative (Spray-cyte; Clay Adams, Parsippany, NJ), and were stained using the modified Papanicolaou method. Cell types were counted as parabasal, intermediate, and superficial, using the criteria of Papanicolaou (17). Keratinized, anucleate cells were considered superficial. One

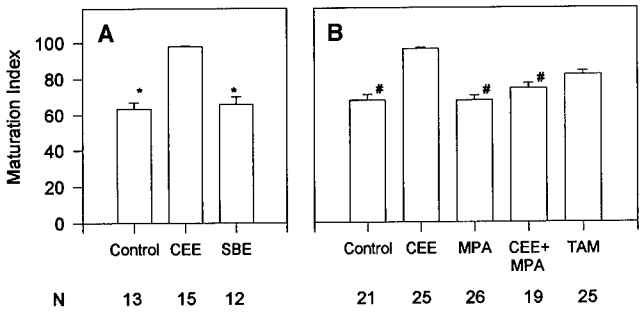


**Figure 1** Vaginal epithelial maturation indices in surgically postmenopausal monkeys described in studies 1 (**A**) and 2 (**B**). Error bars are SEM. Bars with shared symbols are not statistically different at $P \leq 0.05$. Numbers below bars indicate numbers of animals per group. CEE, conjugated equine estrogens; SBE, soybean estrogens; TAM, tamoxifen.

hundred cells per slide were counted and classified. The presence of leukocytes, erythrocytes, and mucus also was noted. Maturation of the epithelium was determined by the maturation index (MI). The MI was calculated by assigning weighting values of 0.2 to parabasal cells, 0.6 to the intermediate cells, and 1.0 to the superficial cells. The percentage of each cell type was multiplied by its assigned value and then the products were summed to give the MI (18). For purposes of comparison with previous reports of vaginal cytology in primates, the karyopyknotic index (KPI) also was calculated. The KPI is the ratio of mature superficial squamous cells to mature intermediate squamous cells, as described by Seier et al. (19).

### Serum Hormone Measurements

Measurements were made of $E_2$ and P during the initial phase of the study to confirm completeness of ovariectomy. Evaluations were made of plasma concentrations of $E_2$ during the treatment phase, using a modification of a commercial kit (Diagnostic Products Corporation, Los Angeles, CA). Samples were taken 4 hours after feeding and dosing. All hormone measurements were carried out at the Comparative Endocrinology Laboratory of the Yerkes Regional Primate Center of Emory University (Atlanta, GA).

Serial serum isoflavone concentrations (genistein and daidzein and their metabolites, genistein sulfate and daidzein sulfate) were measured in a subset of six animals by high-performance liquid chromatography and mass spectroscopy. Isoflavone analyses were performed by Steven Barnes, Department of Pharmacology and Toxicology, University of Alabama at Birmingham. Data were analyzed using one-way analysis of variance, using a $P$ value of 0.05.

## RESULTS

Maturation and karyopyknotic indices are illustrated in Figures 1 and 2. For study 1, maturation indices (mean ± SD) were as follows: controls, 63.4 ± 12.9; conjugated equine estrogens, 98.3 ± 1.2; and soybean estrogens, 66.1 ± 14.5. Karyopyknotic indices were 4.0 ± 13.5, 47.2 ± 35.1, and 1.1 ± 2.3, respectively. For study 2, maturation indices were as follows: controls, 67.9 ± 14.5; conjugated equine estrogens, 96.6 ± 3.9; conjugated equine estrogens combined with MPA, 74.4 ± 11.8; MPA, 67.8 ± 11.7; and tamoxifen, 81.9 ± 16.4. Karyopyknotic indices were 1.7 ± 3.4, 29.7 ± 32.0, 1.4 ± 3.9, 2.8 ± 2.8, and 6.0 ± 10.0, respectively.

In each study, maximal maturational effects on vaginal epithelium were seen in the group given conjugated equine estrogens alone. In study 1, there was a complete lack of maturational effect among animals fed the soybean estrogens diet.

In study 2, the estrogenic effect of conjugated equine estrogens was antagonized by MPA when the two were given in combination. Medroxyprogesterone acetate alone did not enhance epithelial maturation. Tamoxifen was intermediate to the conjugated equine estrogens and control groups in its estrogenic effect on vaginal epithelium. There was no effect of treatment on the presence of blood, mucus, or inflammatory cells.

Mean plasma $E_2$ concentrations over the course of each study were calculated from all available values. For study 1, they were as follows: controls, 3.8 pg/mL (13.95 pmol/L); conjugated equine estrogens, 147 pg/mL (539.64 pmol/L); and soybean estrogens, 0 pg/mL (0 pmol/L). We interpreted the failure to detect $E_2$ in the soybean estrogens–treated animals as meaning that there is no cross-reactivity between $E_2$ and soybean estrogens in the assay. For study 2, plasma $E_2$ concentrations were as follows: controls,

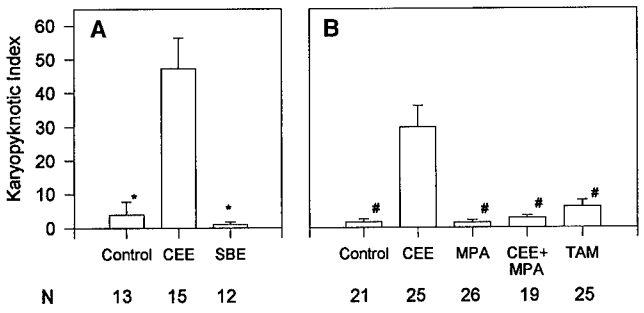


**Figure 2** Vaginal cytologic characteristics as described by karyopyknotic indices in surgically postmenopausal monkeys described in studies 1 (**A**) and 2 (**B**). Error bars are SEM. Bars with shared symbols are not statistically different at $P \leq 0.05$. Numbers below bars indicate numbers of animals per group. See Figure 1 legend for description of abbreviations.

7.0 pg/mL (5.70 pmol/L); conjugated equine estrogens, 96.3 pg/mL (353.52 pmol/L); MPA, 10.6 pg/mL (38.92 pmol/L); conjugated equine estrogens combined with MPA, 109.6 pg/mL (402.34 pmol/L); and tamoxifen, 6.0 pg/mL (22.03 pmol/L). For each study, all groups receiving conjugated equine estrogens were significantly different from other groups at $P < 0.0001$.

Serum phytoestrogen concentrations peaked at 2 to 4 hours postprandially; the maximal concentration of genistein sulfate was 36.62 ± 11.72 nM (mean ± SEM) at 2 hours. Maximal concentrations of daidzein, daidzein sulfate, and genistein were 2.82 ± 2.203, 17.91 ± 5.75, and 17.77 ± 4.38 nM (mean ± SEM), respectively, at 4 hours postprandially.

## DISCUSSION

Conjugated equine estrogens are the most commonly prescribed drug for the prevention or amelioration of symptoms of estrogen deficiency. As expected, conjugated equine estrogens had estrogenic effects on vaginal epithelium, which were antagonized to a modest degree by MPA. Furthermore, our results show that tamoxifen, an estrogen antagonist in normal and neoplastic breast tissue, had estrogenic effects on the vaginal epithelium in macaques that are similar to those seen in women (10). A noteworthy finding in this study is the lack of an estrogenic effect of soybean estrogens on the vaginal epithelium. Evidence is accumulating that soybean estrogens may have favorable estrogenic effects on risk of cardiovascular disease and breast cancer. Beneficial effects on serum lipids (lowering of total plasma cholesterol and increases in high-density lipoprotein cholesterol) have been documented in macaques fed the diets described herein (5). Our data indicate that those changes may not be accompanied by an estrogenic effect on the reproductive tract (5). An important implication of this finding is that soybean estrogens may have a useful spectrum of tissue selectivity. Histologic studies of the effects of soybean estrogens on vagina and uterus will be conducted on these animals at the conclusion of this ongoing trial; preliminary data from a previous pilot study of 14 monkeys are in agreement with the material presented herein (Cline JM, unpublished observations).

The major phytoestrogens in soybean estrogens, genistein and daidzein, bind only weakly to the estrogen receptor, at two to three orders of magnitude lower than the affinity of $E_2$. Their major active serum metabolite, equol, has similar affinity (20). Despite this low receptor affinity, plasma concentrations of soybean estrogens similar to those achieved in this study inhibit tumor cell growth in vitro, an effect independent of the estrogen receptor positivity of the cells (21). Inhibition of tumor growth has been seen also in a number of rodent mammary carcinoma models (22). The potential differential effect of tamoxifen and other nonsteroidal estrogen agonists and antagonists on estrogen-responsive elements of the genome may explain the tissue selectivity of these compounds (23); however, the role of soybean estrogens in this context has not been distinguished from simple differences in receptor affinity as shown by Markiewicz et al (24). The estrogen-receptor–independent, antiproliferative effect of genistein may be mediated through its potent inhibitory effect on tyrosine kinases or via its potent antiangiogenic properties (25); it is likely that both contribute to its antitumor effect in animal models (22).

The future usefulness of selectively estrogenic compounds will depend on more information regarding their lack of classical estrogenicity in the reproductive tract and breast, while preserving estrogenic activity in the prevention of atherosclerosis, osteoporosis, and possibly senile dementia. Our data indicate that soybean estrogens is a selective estrogen, because doses that induce estrogen-like effects in serum lipids do not elicit estrogenic effects on the vaginal epithelium (5). Work is in progress to compare further the mammotrophic and uterotrophic effects of soybean estrogens with those of pharmacologic estrogens, using nonhuman primates in which atherosclerosis, osteoporosis, and cognitive function also are being assessed.

*Acknowledgments.* We are grateful for the support and assistance of Thomas B. Clarkson, D.V.M., in the pursuit of this work; for the assistance provided by Mark Wilson, Ph.D., in the measurement of serum $E_2$ concentrations; and for the technical assistance of Brian McCollough, B.S. We gratefully acknowledge the gift of soy protein isolates for this study from Protein Technologies International, St. Louis, Missouri.

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**Appendix B: Meeting Abstracts**

(copies of items in **bold** are attached)

Cline JM, von Schoultz B, Skoog L, Clarkson TB. Can the cynomolgus macaque be used as a model for studying the effect of exogenous hormones on women? The Seventh International Congress on the Menopause, Stockholm, Sweden, June 21-25, 1993

Cline, JM; Soderqvist, G; von Schoultz, E; Skoog, L; von Schoultz, B Addition of medroxyprogesterone acetate to conjugated equine estrogens in surgically postmenopausal macaques: Divergent effects on mammary and endometrial tissue. Triangle Conference on Reproductive Biology, Research Triangle Park, NC, January 14, 1995

**Divergent effects of hormone replacement on mammary and endometrial tissues of macaques. Cline, JM; Soderqvist, G; Skoog, L; von Schoultz, B Sixth Annual Meeting of the North American Menopause Society, San Francisco, CA, September 1995.**

**Cline JM, Bain FT. Uterine vascular changes indicating prior pregnancy in macaques. Vet Pathol 32:585, 1995.**

**Cline JM, Foth D. Effect of mammalian and plant estrogens, tamoxifen, and medroxyprogesterone acetate on epithelial proliferation in the mammary glands and uteri of macaques. Proceedings, American Association for Cancer Research. 1996;37:284.** ***(meeting handout attached)***

Cline JM, Foth D. Effects of mammalian and plant estrogens, tamoxifen, and medroxyprogesterone acetate on epithelial proliferation in the mammary glands and uteri of macaques. Triangle Conference on Reproductive Biology, Research Triangle Park, NC, January 16, 1996

Cline JM. Endometrial changes induced by estrogens, tamoxifen, progestins, androgens and soy in macaques. Accepted, American College of Veterinary Pathologists, Seattle Washington, December 1996

Cline JM, Soderqvist G, Foth D, Romer T, von Schoultz B. Effects of estrogens, tamoxifen, progestins, androgens and dietary soy onthe mammary gland and endometrium of macaques. Accepted, The Eighth International Congress on the Menopause, Sydney, Australia, November 1996.

# DIVERGENT EFFECTS OF HORMONE REPLACEMENT ON MAMMARY AND ENDOMETRIAL TISSUES OF MACAQUES

Cline, JM[1]; Soderqvist, G[2]; Skoog, L[2]; von Schoultz, B[2]
*[1]Bowman Gray School of Medicine, Winston-Salem, NC, USA; and [2]Karolinska Hospital, Stockholm, Sweden.*

The purpose of this study was to evaluate cancer risk-related markers in a primate model of postmenopausal hormone replacement therapy. Surgically postmenopausal adult female macaques (*Macaca fascicularis*) were given oral conjugated equine estrogens (CEE, n = 22), CEE and medroxyprogesterone acetate (CEE+MPA, n = 21) or no treatment (n = 26), for 30 months. Doses were equivalent to 0.625 mg/woman/day for CEE and 2.5 mg/woman/day for MPA. Mammary and endometrial changes were assessed by morphologic and immunohistochemical means. Tissues were stained for estrogen receptor (ER), progesterone receptor (PR), and the proliferation marker Ki-67 MIB (MIB). **Results:** *In mammary gland* - CEE+MPA induced greater proliferation than CEE, by point counting estimates of lobular size, % epithelium and % of MIB-stained cells (2-8% for CEE+MPA, <1% for others; p=<0.05 vs. controls). ER+ cells declined in the CEE+MPA group (2-4% vs. 7-20% for others, p<0.03). The % of PR+ cells increased with CEE alone (20-30%, p=0.0003), compared to controls and CEE+MPA (3-7%). *In uterus* - Endometrial thickness, MIB staining, and percent glands were greatest in the CEE group. CEE+MPA decreased endometrial thickness (2.04mm vs. 2.6 for CEE alone), and decreased epithelial MIB staining (0.8 vs 1.72%, nsd). **Conclusion:** There is a proliferative response of mammary gland epithelium to CEE+MPA in postmenopausal macaques, in the face of decreased ER and PR, and in contrast to the antagonism of CEE and MPA in endometrium.

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UTERINE VASCULAR CHANGES INDICATING PRIOR PREGNANCY IN MACAQUES. J.M. Cline, F.T. Bain. Department of Comparative Medicine, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157

In ongoing studies of mammary cancer risk, the need arose to retrospectively distinguish nulliparous and parous macaques without the benefit of a reproductive history. In order to address this problem, histologic sections of uteri were examined from 92 female macaques of known parity. Animals were of 3 species; *Macaca fascicularis* (n=36), *M. mulatta* (n=51) and *M. nemestrina* (n=5). Animals were colony-bred, nonpregnant, sexually mature (≥4 years of age),and had a known reproductive history. Animals dying <1 month after their last delivery were excluded. The mean age was 9.16 years (sd 3.30, range 8.5-16.7) for the 67 parous animals and 5.44 (sd 2.17, range 4.75-12.3) for the 26 nulliparous animals examined. Among parous animals, mean parity number was 2.5 (sd 1.8, range 1-7). Mean interval from last delivery was 18.45 months (sd 13.42, range 1-78). Sections of all uteri were examined independently by 2 veterinary pathologists blinded to the animals' reproductive histories. Uteri were classified as parous or not by the presence or lack of perivascular extracellular matrix deposits around uterine vessels. Differences of opinion were resolved by consensus. Matrix deposit extent was graded (1-3). Using this criterion, 84/92 animals (91%) were correctly identified as to parity status. Two of 25 nulliparous animals (2% of the total) were incorrectly identified as parous; 6/67 parous animals (7% of the total) were incorrectly identified as nulliparous. Interval from parity in the 6 misclassified parous animals was 6 to 30 months (mean 19.8). Matrix deposits persisted 78 months in one animal. There was a tendency for grade to increase with number of births and decrease with time after the last birth. Age did not independently correlate with matrix grade. We conclude that the presence of uterine perivascular matrix deposits is a reliable indicator of prior pregnancy in macaques, but that parity number and time since last pregnancy cannot be estimated from this feature.

## 158

UTERINE INFARCTS IN *MACACA FASCICULARIS*. F.Bain, J.Cline, J.Fikes, C.Carlson, M.O'Sullivan, M.Jayo.
The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27157-1040

Uterine infarcts are unreported in cynomolgus monkeys (*Macaca fascicularis*), and there are few reports in the human medical literature. We identified uterine infarcts in 8 out of 98 cynomolgus monkeys necropsied between November 1993 and April 1995. The infarcts were visible on the serosal surface of the uterus in 2 monkeys, and were seen only in cut section in the remainder. They occupied up to 50-60% of the cross-sectional area of transverse sections. Histologically, there were well-demarcated regions of endometrial and myometrial necrosis and hemorrhage. All monkeys had evidence of prior pregnancies, either historically or by the presence of altered matrix within the tunica media of uterine vessels. Five monkeys had been ovariectomized for experimental protocols 2 years or less prior to death and 3 were intact. Common clinical findings included severe trauma due to fighting or severe diarrhea, both of which resulted in signs of hypovolemia. Pathologic findings included cutaneous or skeletal muscle necrosis (4 monkeys), enterocolitis (3 monkeys), and intestinal amyloidosis (1 monkey). Other changes included pulmonary edema or diffuse alveolar injury in 3 monkeys, and fibrin deposition in multiple organs in 4 monkeys. Uterine infarcts in cynomolgus monkeys appear to be associated with injuries or diseases causing hypovolemia, suggesting that dehydration, hypovolemic shock, and possibly disseminated intravascular coagulation may be involved in the pathogenesis of this lesion. Prior pregnancy also may be a predisposing factor as we have not seen this lesion in nulliparous animals.

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A MOUSE MODEL OF ENDOMETRIOSIS: TRANSPLANTATION OF HUMAN ENDOMETRIAL CELLS TO THE PERITONEAL CAVITY OF SCID MICE. R.L. Donnell, S. Van Meter, N.B. Upadhyaya, L. Munson. Department of Pathology, College of Veterinary Medicine, University of Tennessee, Knoxville,TN 37901

Endometriosis in humans arises from the ectopic peritoneal growth of uterine epithelium. Understanding the pathogenesis of this disease would be greatly enhanced by a suitable animal model. SCID mice were chosen for this model because they lack normal lymphocyte-mediated rejection of heterologous tissues. Primary cultures of human endometrial epithelial cells, derived from tissue explants, were placed in the peritoneal cavities of female SCID mice as cell suspensions or cells supported on various substrata. After 2 weeks, the mice were examined for gross and histologic evidence of implantation and graft survival. Transplanted cells were identified microscopically, and the human epithelial origin of these cells was confirmed by immunohistochemistry. Cells supported on substrata were accompanied by fibroplasia and neovascularization in the mesometrium and mesentery. These results indicate that cultured human endometrial epithelium transplanted to the peritoneal cavity of SCID mice remain viable for 2 weeks and could provide an improved model for endometriosis in women.

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SPONTANEOUS ENDOMETRIOSIS IN RHESUS MACAQUES IN A LARGE BREEDING COLONY. M. Rippy, R. Lee, J. Bernal, S. Pearson, and T. Kuehl.
Lilly Research Laboratories, Greenfield, IN 46140 and HRP, INC.,Texas Primate Center, Alice, TX 78333

Occurrence of spontaneous endometriosis has been reported in several species of nonhuman primates and is associated with infertility in human females. Breeding records, health status, and body condition from rhesus macaques scheduled for culling from a large breeding colony were examined. Macaques (103) in good health and with a history of no live births within the previous 3 years were bled for determination of CA-125 antigen levels. Macaques (39) were chosen for laparoscopy and pelvic ultrasonography after assessment of age, breeding history, body condition, rectal palpation, and CA-125 levels. Endometriosis was diagnosed in 12 of the 39 macaques (31%) examined by laparoscopy. Of these 12 macaques, 7 macaques were biopsied and the diagnosis confirmed by histopathology. Additionally, fibrous adhesions involving pelvic organs in the absence of endometriotic sites were present in 5 macaques and 3 of these 5 macaques had no history or evidence of a previous surgery. The animals with endometriosis were generally thin and ranged in age from 14 to 25 years (avg.= 18 years old). Of the 12 documented cases of endometriosis, the stages were minimal, 6; mild, 4; moderate, 1; and severe, 1. Common sites of the disease included the serosal surface of the urinary bladder and uterus, the pelvic cul de sac, and the broad ligaments of the uterus. None of the animals examined by pelvic ultrasonography had uterine leiomyomas.

# EFFECTS OF MAMMALIAN AND PLANT ESTROGENS, TAMOXIFEN, AND MEDROXYPROGESTERONE ACETATE ON EPITHELIAL PROLIFERATION IN THE MAMMARY GLANDS AND UTERI OF MACAQUES

Cline, JM[1]; Foth, D[2]. [1]Department of Comparative Medicine, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC, and [2]Ernst-Moritz-Arndt University, Greifswald, Germany.

**Introduction**

The macaque model offers a unique opportunity for the study of hormonal and dietary effects on women's health, since it enables parallel evaluation of the effects of treatments on endometrium, mammary gland, cardiovascular system, bone, and behavioral endpoints in a primate.

Recent work at Bowman-Gray has focused on the use of this animal model of post-menopausal hormone treatment effects for the assessment of intermediate markers of cancer risk in the breast and endometrium. Macaques are similar to women in many aspects of reproductive physiology and anatomy, and we believe they provide a good model for the evaluation of mammary gland and endometrial regulation.

**Methods**

In two recent studies of hormonal replacement therapy and its alternatives, adult, surgically postmenopausal female macaques (*Macaca fascicularis*) were treated continuously with either estradiol (E2), isoflavone-enriched soy isolate (Soybean estrogens: SBE), or E2+SBE for 6 months (Study 1); or with either conjugated equine estrogens (CEE), medroxyprogesterone acetate (MPA), the combination of CEE+MPA, or tamoxifen for 2 years (Study 2). Test compounds were adminstered in the diet, at doses equivalent on a caloric basis to 0.625 mg/woman/day for CEE, 2.5 mg/woman/day for MPA, 20 mg/day for Tamoxifen, 1 mg/woman/day for estradiol, and 148 mg/woman/day for soy isoflavones. Proliferation was assessed by histopathologic, morphometric and immunohistochemical means in endometrium and mammary gland.

**Results**

Endometrium

- Increases in endometrial thickness and gland area (as a percentage of total endometrial area) were induced by estrogens, tamoxifen, CEE, E2, and E2+SBE (see *Endometrial Measurements bar graph*).
- Endometrial hyperplasia was simple, cystic or irregular; clear-cut adenomatous hyperplasia was not seen. MPA induced stromal hyperplasia (see *Subjective Evaluation tables and photographs*).
- Tamoxifen induced the greatest degree of endometrial hyperplasia.
- Two endometrial polyps were observed in tamoxifen-treated animals.
- Morphometric changes were accompanied by increased Ki67 staining in the CEE and E2 treated groups, but not in the tamoxifen-treated group (see *Cell Proliferation bar graphs*).
- The effects of E2 were partially antagonized by SBE (manifested as decreased Ki-67 staining; see *Study 1 Cell Proliferation bar graph*).

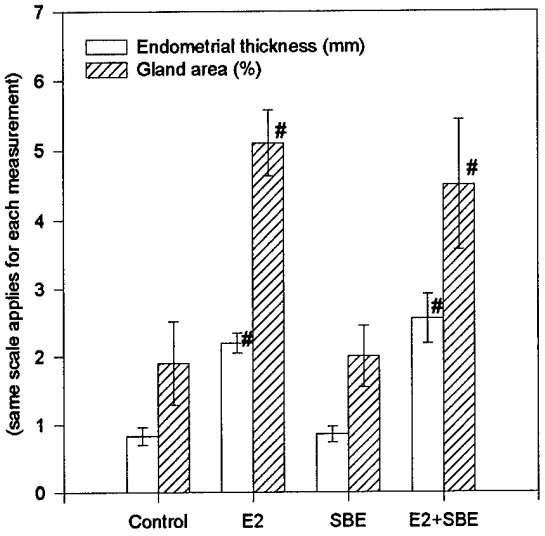
Mammary Gland

(*see Cell Proliferation and Mammary Gland Measurements bar graphs*)

- Mammary gland proliferation was induced by CEE, CEE+MPA, E2, and E2+SBE
- Mammary hyperplasia consisted of diffuse lobular enlargement; ductal hyperplasia and atypia were not seen (see *Subjective Evaluation tables and photographs*).
- Proliferative changes were maximal in animals given CEE+MPA.
- Morphometric and immunohistochemical measures of proliferation were in agreement in this tissue.
- The effects of E2 were antagonized by SBE in the mammary gland.
- Tamoxifen did not induce mammary gland proliferation.

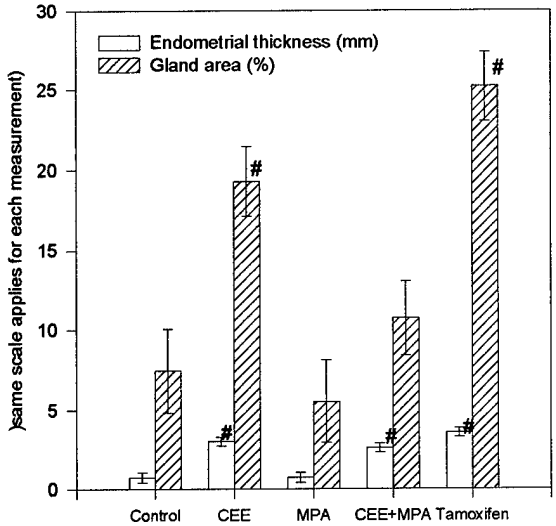
*In all graphs, groups differing from controls at p<0.05 are indicated by "#".*

## Study 1 - Endometrial Measurements



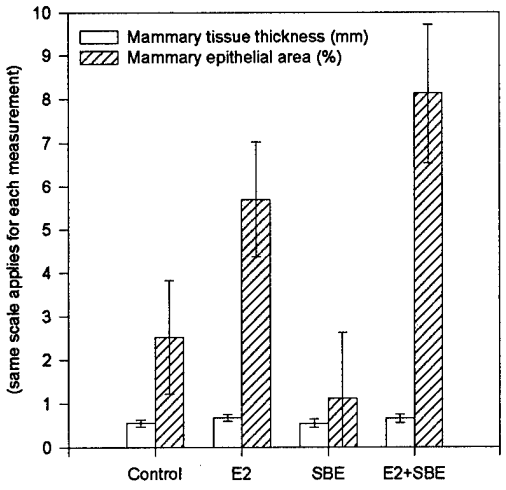

Estradiol induced endometrial hyperplasia. Soy phytoestrogens alone (SBE) did not induce endometrial hyperplasia. Morphologic measures did not detect an interaction between E2 and SBE.

## Study 2 - Endometrial Measurements



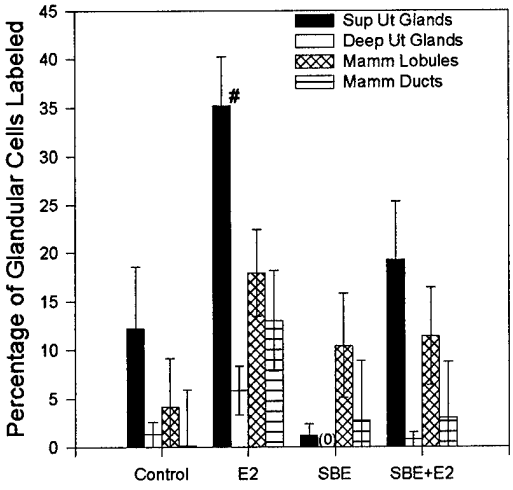

Conjugated estrogens and tamoxifen induced endometrial hyperplasia. The effect of CEE was antagonized by MPA.

## Study 1 - Mammary Gland Measurements



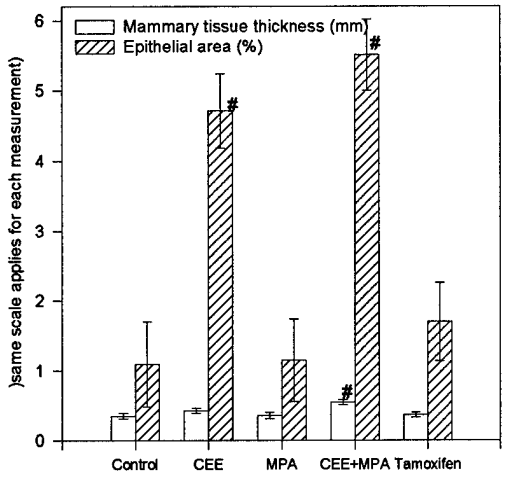

Estradiol induced mammary gland hyperplasia. Soy phytoestrogens alone (SBE) did not induce mammary gland hyperplasia. Morphologic measures did not detect an interaction between E2 and SBE.

## Study 1 - Cell Proliferation (Ki67 Staining)



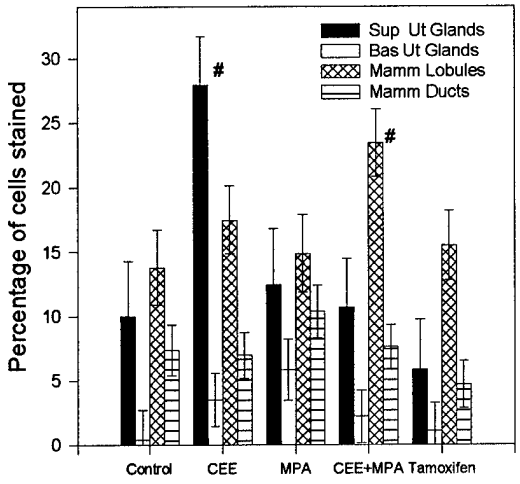

Soy antagonizes Ki-67 proliferation marker induction by estrogen in mammary gland and endometrium.

## Study 2 - Mammary Gland Measurements




MPA did not antagonize the effect of conjugated estrogens on mammary gland, in contrast to the situation in the endometrium.

## Study 2 - Cell Proliferation (Ki67 Staining)




Maximal induction of the proliferation marker Ki67 MM1 in uterus resulted from CEE treatment. For the mammary gland, CEE+MPA maximized proliferation. In the case of tamoxifen, the immunohistochemical marker indicates little proliferation, despite the histologic appearance of hyperplasia.

**Conclusions**

These results indicate that in this primate model,

1. **The addition of MPA to CEE treatment exerts divergent effects on the endometrium and mammary gland;**
2. **that tamoxifen is strongly uterotrophic, but did not increase Ki-67 expression as did CEE and E2; and**
3. **that soybean estrogens may have antiproliferative effects in the endometrium and mammary gland of E2-treated animals.**

These findings have relevance to the potential risk associated with combined CEE+MPA for hormonal replacement therapy; provide evidence for an unusual pattern of endometrial proliferation in animals treated with tamoxifen; and suggest that soy supplementation may have a beneficial antagonistic effect on endometrial and mammary proliferation when given with exogenous estrogen.

Supported by USAMRAA DAMD17-94-J-4201, NHLBI HL-38964 and HL-45666.